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Efficiency of neem (*Azadirachta indica*) as a nitrification inhibitor under laboratory conditions

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ABSTRACT

The use of nitrification inhibitors is a technology that can increase the efficiency of nitrogen fertilization, and synthetic and natural products can be used for this purpose. However, the use of these products is still poorly studied in tropical regions. The objective of this work was to evaluate the nitrification of nitrogen, under laboratory conditions, with urea covered with doses of neem leaf or cake, using DMPP (3,4-dimethylpyrazole phosphate) for comparison. The treatments consisted of a 2 x 4 + 2 factorial scheme, with the first factor represented by neem sources (leaf and cake), used to cover the urea and the second factor by neem doses, these doses being 0, 10 , 20 and 40% of the total applied N (200 mg kg-1, using urea as a N source) and two additional treatments: control (soil without nitrogen fertilizer) and urea + DMPP. Ammonium and nitrate contents were evaluated right after incubation (time 0) and on the 7th, 15th, 30th, 60th and 90th day after the start of incubation. For the ammonium content in the soil, there was influence of source and dose on the fifteenth day of incubation, with linear increments for both sources; and for nitrate levels, there was a significant effect (p<0.01) of neem doses on the 7th and 15th days of incubation, with a reduction in levels depending on the doses for the two sources tested. Significant differences (p<0.01) were found between additional and factorial treatments and between additional treatments for ammonium levels from the 7th to the 90th day of incubation. There was no significant interaction, in any of the dates, between neem sources and doses for ammonium and nitrate contents in the soil. Covering the urea with neem leaf and cake was efficient in reducing the levels of nitrate in the soil up to 15 days after incubation, resulting in higher levels of ammonium in the soil until this date. However, DMPP was more efficient in reducing nitrification, promoting inhibition up to 60 days of incubation.

Keywords: ***Nitrogen; Nutrient Management; urea.***

Eficiência do nim (*Azadirachta indica*) como inibidor de nitrificação em condições de laboratório

RESUMO

A utilização de inibidores de nitrificação é uma tecnologia que pode aumentar a eficiência da adubação nitrogenada. Porém, a utilização destes produtos ainda é pouco estudada em regiões tropicais. O objetivo do trabalho foi avaliar a nitrificação do nitrogênio, em condições de laboratório, com ureia recoberta com doses de folha ou torta de nim, utilizando o DMPP (Fosfato de 3,4-dimetilpirazol) para comparação. Os tratamentos foram constituídos de um esquema fatorial 2 x 4 + 2, com o primeiro fator representado por fontes de nim (folha e torta), utilizadas para recobrir a ureia e o segundo fator de doses de nim, sendo estas doses de 0, 10, 20 e 40% do total de N aplicado (200 mg kg^{-1}) e dois tratamentos adicionais: testemunha (solo sem fertilizante nitrogenado) e ureia + DMPP. Avaliou-se os teores de amônio e nitrato logo após a incubação (tempo 0) e no 7º, 15º, 30º, 60º e 90º dia. Para o teor de amônio no solo, houve influência de fonte e dose no décimo quinto dia de incubação, com incrementos lineares para as duas fontes; e para os teores de nitrato, houve efeito significativo (p<0,01) das doses de nim, no 7º e 15º dia de incubação, com redução dos teores em função das doses para as duas fontes testadas. Observou-se diferenças significativas (p<0,01) entre os tratamentos adicionas e o fatorial e entre tratamentos adicionais, para os teores de amônio do 7º a 90ªdia de incubação. Não houve interação significativa entre fontes e doses de nim para os teores de amônio e nitrato no solo. O recobrimento da ureia com folha e torta de nim foi eficiente em reduzir os teores de nitrato no solo até 15 dias após a incubação, resultando em maiores teores de amônio no solo. Todavia, o DMPP mostrou-se mais eficiente em reduzir a nitrificação, promovendo inibição até os 60 dias de incubação.

Palavras-chave: ***Nitrogênio; Manejo de Nutrientes; ureia.***

RESUMEN

Eficiencia del neem (*Azadirachta indica*) como inhibidor de la nitrificación en condiciones de laboratorio

El uso de inhibidores de la nitrificación es una tecnología que puede aumentar la eficiencia de la fertilización con nitrógeno. Sin embargo, el uso de estos productos aún está poco estudiado en las regiones tropicales. El objetivo del trabajo fue evaluar la nitrificación del nitrógeno, en condiciones de laboratorio, con urea recubierta con dosis de hoja o torta de neem, utilizando como comparación DMPP (fosfato de 3,4-dimetilpirazol). Los tratamientos consistieron en un esquema factorial 2 x 4 + 2, siendo el primer factor representado por las fuentes de neem (hoja y torta), utilizadas para cubrir la urea y el segundo factor por las dosis de neem, siendo estas dosis 0.10, 20 y 40% de el N total aplicado (200 mg kg-1) y dos tratamientos adicionales: testigo (suelo sin fertilizante nitrogenado) y urea + DMPP. Los niveles de amonio y nitrato se evaluaron inmediatamente después de la incubación (tiempo 0) y los días 7, 15, 30, 60 y 90. Para el contenido de amonio en el suelo hubo influencia de la fuente y la dosis al decimoquinto día de incubación, con incrementos lineales para ambas fuentes; y para los niveles de nitrato, hubo un efecto significativo (p<0,01) de las dosis de neem, en el día 7 y 15 de incubación, con una reducción de los niveles dependiendo de las dosis para las dos fuentes probadas. Se observaron diferencias significativas (p<0,01) entre los tratamientos adicional y factorial y entre tratamientos adicionales, para los contenidos de amonio del 7º al 90º día de incubación. No hubo interacción significativa entre las fuentes de neem y las dosis para los niveles de amonio y nitrato en el suelo. Cubrir la urea con hojas de neem y torta fue eficaz para reducir los niveles de nitrato en el suelo hasta 15 días después de la incubación, lo que resultó en niveles más altos de amonio en el suelo. Sin embargo, el DMPP demostró ser más eficiente para reducir la nitrificación, promoviendo la inhibición hasta los 60 días de incubación.

Palabras clave: ***Nitrógeno; Manejo de Nutrientes; urea.***

Introduction

Urea is the main form of nitrogen fertilizer used in Brazilian agriculture (ESPINDULA *et al*., 2013). Such fertilizer, after being distributed onto the soil, may lose ammonia due to volatilization in the hydrolysis process (MALHI *et al.*, 2001; RAIJ, 2011). Furthermore, other processes cause N losses to the environment. After applying urea onto the soil, nitrification will take place, transforming NH_4^+ into NO_3^- by microorganisms through biological oxidation (SUBBARAO *et al.*, 2006). Then, this nitrate is susceptible to leaching, and there is the possibility of loss through denitrification, which occurs when NO_3^- transforms into N_2 (NO_3^- → NO_2 → NO → N_2O → N_2). N_2O may also be released to the atmosphere as a result (SUBBARAO *et al.*, 2006).

Denitrification takes place especially in poor drained soils in anaerobic conditions. The N loss rate through denitrification is higher when the soil is very moist (DOBBIE & SMITH, 2001). Such rate may be even higher among clay soils (CAMERON, DI & MOIR, 2013). Due to these factors, the restoration efficiency of N applied rarely surpasses 50% (ABBASI, HINA & TAHIR, 2011). Losses through volatilization may reach 78% of the total N applied. This whole process may be intensified when urea is applied over vegetal residues, which will cause urease activity to increase (LARA CABEZAS, KORNDORFER & MOTTA, 1997).

One of the technologies recently tested to enhance the efficiency of nitrogen fertilizers is the use of nitrification inhibitors. These inhibitors prevent nitrification by halting the action of nitrosomonas – which causes a delay in the conversion of ammonium into nitrate in four to ten weeks depending on the soil's temperature and pH (BUNDICK *et al.*, 2009). By reducing nitrification until the plant is fully grown, it is possible to increase NO_3^- absorption (SUBBARAO *et al.*, 2006).

Nitrapyrin and DICY (dicyandiamide) are the most known and tested nitrification inhibitors, along with the recent 3,4-dymethylpyrazole phosphate (DMPP) (GOOS & JOHNSON, 1999; CALDERON *et al.*, 2005; ISLAM, CHEN & WHITE, 2007). According to Chen *et al*. (2008), DMPP is one of the inhibitors with the highest potential of reducing N loss in the soil. Abbasi, Hina & Tahir (2011)

report that nitrification inhibitors are generally expensive. According to all authors previously cited, products derived from more common salts or from vegetal materials tend to be cheaper and more accessible, and may be as efficient as synthetic inhibitors, e.g. neem.

Neem (*Azadirachta indica*) is originally from southeast Asia (PATRA *et al.*, 2002). Belonging to the same family as the Amazonian mahogany, *Meliaceae*, neem produces excellent wood. This ancient tree is distributed throughout tropical and subtropical Africa, Australia and the Americas. It possesses a high number of secondary metabolites with biological activity – azadirachtin being the most important (VIANA & PRATES, 2003; VILELA, 2008; BRASIL, 2013).

Because of such properties, neem is used to manufacture insecticides, medicines and cosmetics. The plant has also the ability to inhibit nitrification, due to the chemical composition found in its leaves, seeds and fruits, namely azadirachtin, nimbin, nimbinin and nimbidin (PATRA *et al.*, 2002; BRASIL, 2013). Abbasi, Hina & Tahir (2011) verified that neem cake, calcium chloride and sodium thiosulfate, deployed 50 days after incubation reduced nitrification in 54%, 64% and 69% respectively under laboratory conditions.

This research seeks to evaluate the efficiency of neem doses as nitrification inhibitors under laboratory conditions when compared to DMPP.

Material and methods

The experiment took place at the Soil and Fertilizers Department Laboratory of Sao Paulo State University, Jaboticabal campus. The soil used was a dystrophic red latosol with medium texture from the region of Jaboticabal, classified accordingly to meet Empraba (2013) criteria. The sample was collected from a superficial layer (0-20 m depth), air-dried and sifted with a 6 mm mesh sieve. It was then classified for fertility purposes according to methods described by Raij *et al*. (2001) and to soil granulometric analysis (EMBRAPA, 1997).

Results found were: pH ($CaCl_2$) 4.1; organic matter (OM) = 23 g dm^{-3}; P (resin) = 4 mg dm^{-3}; K, Ca, Mg, H+Al and cation-exchange capacity (CEC) = 0.4; 7; 3; 58; 68.4 $mmol_c$ dm^{-3}; base saturation (V%) = 15; clay, silt and sand = 260, 30

and 710 g kg^{-1} respectively. Limestone (CaO = 45%; MgO = 20%; TNRP = 90%) was applied aiming to increase base saturation to 60%. After mixing limestone to the soil, all samples were put to incubate inside polyethylene bags for 30 days with soil moisture at 80% of water retention capacity so that neutralizing reactions could occur.

The treatments were based on a factorial system of 2 x 4 + 2, where the first factor represented neem sources (leaves powder and neem cake) used to cover urea, the second factor represented doses of neem – namely 0, 10%, 20% and 40% of the total amount of N applied (200 mg kg^{-1}, using urea as N source), plus two additional treatments: witness (soil without nitrogen fertilizer) and urea + DMPP. The experimental lining was totally random, with three repetitions. Each lot received 100 mg kg^{-1} of phosphorus and potassium in the forms of Ca phosphate monobasic (p.a.) and potassium sulfate (p.a.).

The neem leaves were washed with water and detergent, rinsed in running water, immersed in a solution of HCl 0.1 mol L^{-1} and then put under distilled water. Next, they were placed in a greenhouse with forced ventilation at 65 ºC until they reached constant mass and crushed with a Wiley mill. Neem cake is the leftover obtained by extracting oil from seeds. The material was sifted with a 0.86mm mesh sieve, and thicker parts were discarded. It was then put to dry in a greenhouse with forced ventilation at 65 ºC until it reached constant mass. All chemical analyses used to determine concentrations of N were performed according to the methodology described by Bataglia *et al.* (1983). Concentrations of N within leaves and the neem cake were 25.5 and 18.9 g kg^{-1} respectively.

To help cover urea with neem sources, vegetal oil was used (4 g kg^{-1}). After mixing the oil with urea, the mixture was shaken for 15 minutes carefully so that granules would not brake. Later, neem cake and leaves powder were added to the tubes and shaken once more for 15 minutes, causing urea granules to equally line. Where no doses of neem were deployed, vegetal oil was absent. A pre-test of 15 days was performed before running this experiment in which common urea was compared to the oil-mixed urea. There was no significant difference in ammonium and nitrate levels ($p<0.05$), showing that the vegetal oil did not influence the nitrification process.

The experimental units consisted of polyethylene cups of 120 ml, with a four-holed lid to allow oxygenation. For each evaluation date, 30 units were utilized – 180 in total. To settle the experiment, 70 g of soil were weighted, adding potassic and phosphatic fertilizers uniformly mixed.

After placing the soil into the containers, all treatments were put into the incubator. 100 g of each covered urea was diluted in 100 ml of water. From this dilution, a quantity of water equal to the respective dose of N (200 mg kg^{-1}) was removed and distributed onto the soil. Next, water was added to elevate the soil moisture to 60% of water retention. Such capacity had been previously established as water retained at a 60 cm water column tension.

The soils were kept in a no-light environment at 25 ºC, with average variation of ± 1.5 ºC. The moisture was adjusted every two days, and the tubes were weighted. Distilled water was added (if there was a loss superior to 0.05 g) in a careful manner to avoid soil shaking.

The ammonium and nitrate levels were evaluated right after the incubation (time 0) and on the 7^{th}, 15^{th}, 30^{th}, 60^{th} and 90^{th} days after the incubation began. To do so, a sample of 5 g of moist soil was removed, homogenized in 50 ml of KCl mol l^{-1} and then shaken for 60 minutes in a mechanical shaker. After this process, the solution remained resting for 30 minutes, and 25 ml from the floating material was taken. Adding 0.2 g of MgO determined ammoniacal N ($N\text{-}NH_4^+$) using a steam distiller of the semi-micro Kjeldhal type. When the sample finally cooled down, 0.2 g of Devarda's alloy was added so that new a distillation process and nitric N determination ($N\text{-}NO_2^-$ + $N\text{-}NO_3^-$) could take place (CANTARELLA & TRIVELIN, 2001). A second soil sample of 5 g was removed to determine soil moisture and dried in a greenhouse at 110 ºC until it reached constant mass.

Nitrification inhibition was calculated based on a formula proposed by Sahrawat (1980):

$$IN\ (\%) = \left(NU - \frac{NUI}{NU}\right) X\ 100$$

where IN (%) = percentage of nitrification inhibition; NU = concentration of nitrate in the soil with common urea; and NUI = concentration of nitrate in the soil with urea + nitrification inhibitor.

Data was analyzed regarding variance (for main and interaction purposes) and polynomial regression when it was verified that neem doses had had significant effect. When there was substantial influence of sources of neem, a Tukey's test ($p<0.05$) was then performed. All analyses were made using a statistic software called AgroEstat (BARBOSA & MALDONADO JÚNIOR, 2015). Adjustments for ammonium and nitrate levels in the soil in function of time were made through regression models. Another software, the Sigmaplot® 10.0, was used, adopting the equation with the highest determination coefficient (R^2) that best represented the phenomenon.

Results

It was noticed that doses and sources only affected ammonium levels on the 5th day of incubation (**Table 1**). Neem doses significantly affected nitrate levels ($p<0.01$) on the 7th and 15th days of incubation (**Table 2**). Substantial differences in ammonium levels ($p<0.01$) were also verified between additional and factorial treatments, as well as between additional treatments on the 7th and 90th days of incubation (**Table 1**). In the same period, there was also a difference in nitrate levels between additional and factorial treatments, while additional treatments only showed substantial differences on the 90th day of incubation ($p<0.01$) (**Table 2**). No interaction between doses and sources of neem affecting ammonium and nitrate levels in the soil was observed through the entire duration of this experiment (**Tables 1 and 2**).

Figure 1 shows that ammonium concentrations, on the 15th day of incubation, linearly increased in function of neem doses. Additionally, the usage of neem cake covering urea resulted in higher concentrations of ammonium if compared to leaves powder.

Table 1. Statistic analysis of ammonium levels in the soil during 90 days of incubation.

Ammonium, mg kg^{-1}						
Fertilizer	0	7º	15º	30º	60º	90º
Neem fountain (F)						
Leaf	26,03	144,99	120,06	16,89	9,77	8,61
Cake	24,04	149,48	130,8	18,87	6,41	5,49
F	0,43^{NS}	0,96^{NS}	6,95*	0,46^{NS}	1,86^{NS}	2,01^{NS}
Doses of neem (D)						
0%	24,52	143,09	115,32	24,11	7,44	7,11
10%	25,46	150,21	124,35	15,95	7,89	7,30
20%	25,34	151,77	128,59	16,89	10,12	6,8
40%	24,8	143,87	133,45	14,57	6,91	6,9
Teste F	0,02^{NS}	0,92^{NS}	3,57*	2,11^{NS}	0,33^{NS}	0,01^{NS}
F x D	0,25^{NS}	0,45^{NS}	1,62^{NS}	0,04^{NS}	0,12^{NS}	0,24^{NS}
In between additional						
Control	23,12	23,35	22,32	6,34	10,43	4,83
Urea + DMPP	24,35	144,84	148,63	164,35	88,09	72,82
F	0,04^{NS}	175,38**	240,08**	726,17**	248,02**	238,05**
Additional vs factorial	23,73	84,09	85,47	85,34	49,26	38,82
	25,03	147,24	125,43	17,88	8,09	7,05
F	0,15^{NS}	151,57**	76,86**	423,56**	223,07**	168,38**
CV (%)	29,99	8,35	8,5	22,29	36,99	40,26

**Significant at 1%; * Significant at 5% probability. NS not significant.

Table 2. Statistic analysis of nitrate levels in the soil during 90 days of incubation.

Nitrate, mg kg^{-1}						
Fertilizer	**0**	**7º**	**15º**	**30º**	**60º**	**90º**
Neem fountain (F)						
Leaf	47,34	52,56	75,24	174,19	192,05	203,19
Cake	47,4	54,43	72,71	170,12	190,39	205,63
F	0,00^{NS}	1,12 ^{NS}	2,41 ^{NS}	0,92^{NS}	0,05^{NS}	0,24^{NS}
Doses of neem (D)						
0%	46,68	58,55	81,47	171,14	188,44	201,44
10%	44,09	54,28	76,86	178,73	194,99	208,29
20%	48,95	51,40	70,28	168,18	191,03	201,17
40%	49,78	49,74	67,29	170,58	190,43	206,74
Teste F	0,32^{NS}	4,72**	15,44**	1,67^{NS}	0,15^{NS}	0,54^{NS}
F x D	0,18^{NS}	0,74^{NS}	1,51^{NS}	0,82^{NS}	0,38^{NS}	1,51^{NS}
In between additional						
Control	46,59	43,08	49,8	69,42	75,87	80,88
Urea + DMPP	43,69	42,71	48,14	64,86	101,37	161,71
F	0,10^{NS}	0,01^{NS}	0,26^{NS}	0,29^{NS}	3,22^{NS}	66,53**
Additional vs factorial	45,14	42,89	48,97	67,14	88,62	121,96
	47,38	53,49	73,98	172,15	191,22	204,41
F	0,20^{NS}	28,51**	188,68**	492,93**	166,89**	225,10**
CV (%)	23,53	8,47	5,78	6,85	10,19	6,46

**Significant at 1%; * Significant at 5% probability. NS not significant.

Nitrate levels decreased in function of neem doses on the 7th and 15th days of incubation (**Figure 2**). On the 15th day, it was not necessary to apply a

dose higher than 20% of neem cake to cover urea, since the estimated nitrate levels with the equation (quadratic model) for doses of 20% and 40% were respectively 68.38 and 67.58 mg kg^{-1} (**Figure 2-A-B**).

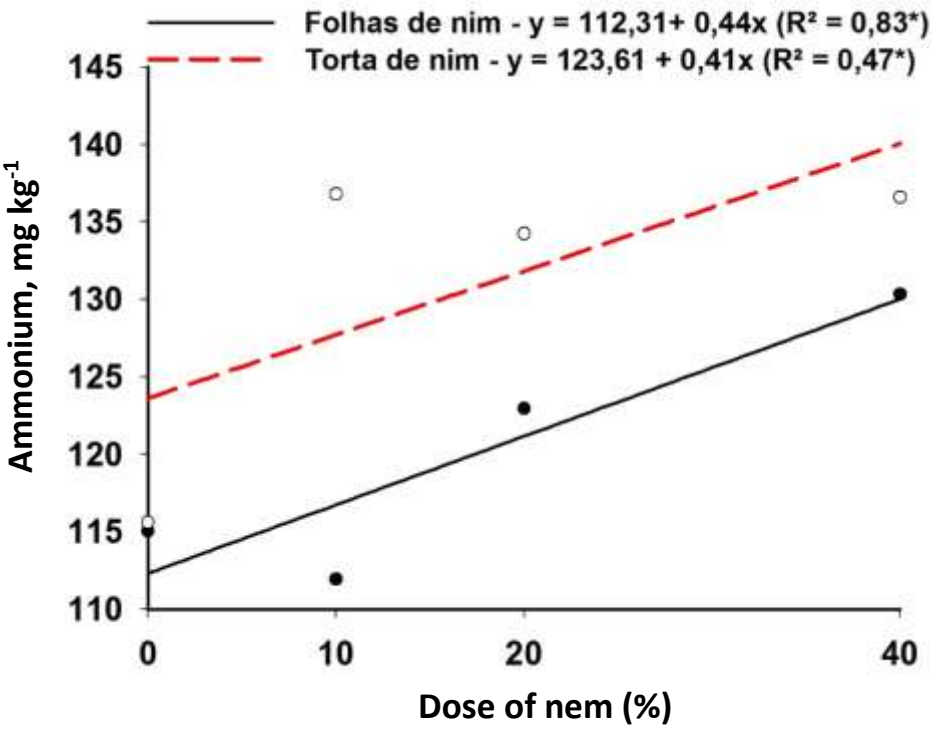

Fig. 1. Ammonium concentration in the soil on the 15th day of incubation in function of doses and sources of neem used to cover urea.

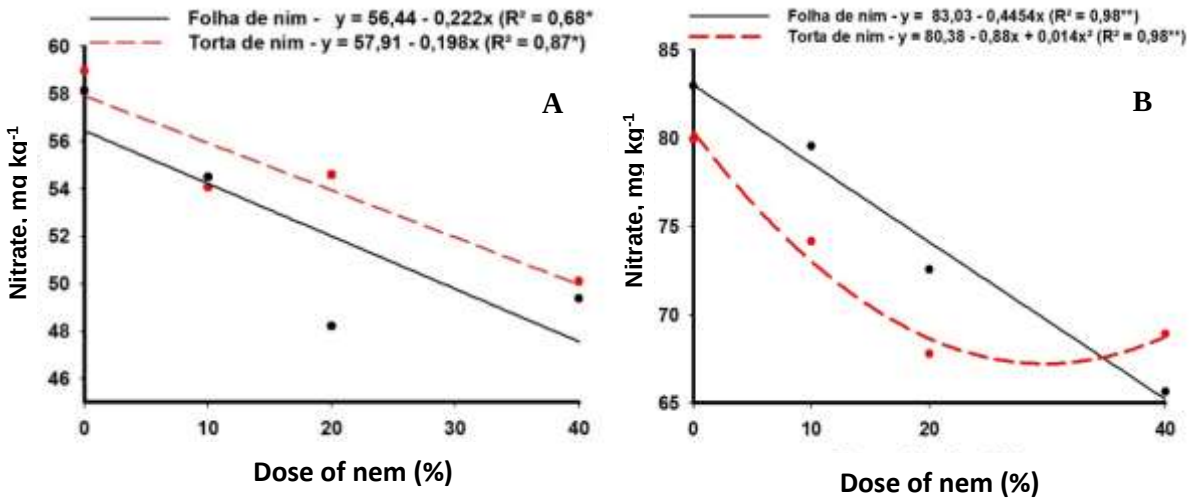

Fig. 2. Nitrate concentration in the soil in function of sources and doses of neem used to cover urea on the 7th (A) and 15th (B) days of incubation.

Treatments injected with nitrogen had such component converted into ammonium by the 7th day (**Figure 2-A**). All other treatments, by the 30th day, had nitrogen entirely converted into nitrate. For urea + DMPP treatments, nitrogen remained as ammonium, reaching full nitric form only on the 90th day of incubation (**Figure 2-A-B**).

The percentage of nitrification inhibition on the 7th day of incubation was 6%, 17% and 15% for treatments using urea and neem leaves, and 8%, 7% and 14% for the ones using neem cake for each dose of 10%, 20% and 40% respectively. On the 15th day, results showed 4%, 12% and 21% for treatments using urea and neem leaves, and 7%, 15% and 14% for the ones using urea and neem cake with the same previous scheme of doses. In the treatment using urea + DMPP, inhibition percentages were 26%, 42%, 63%, 47% and 21% on the 7th, 15th, 30th and 90th days of incubation respectively. DMPP is clearly superior to neem when inhibiting nitrification, keeping most of the nitrogen in ammoniacal form until the 60th day of incubation (**Tables 1 and 2, Figure 3-A-B**).

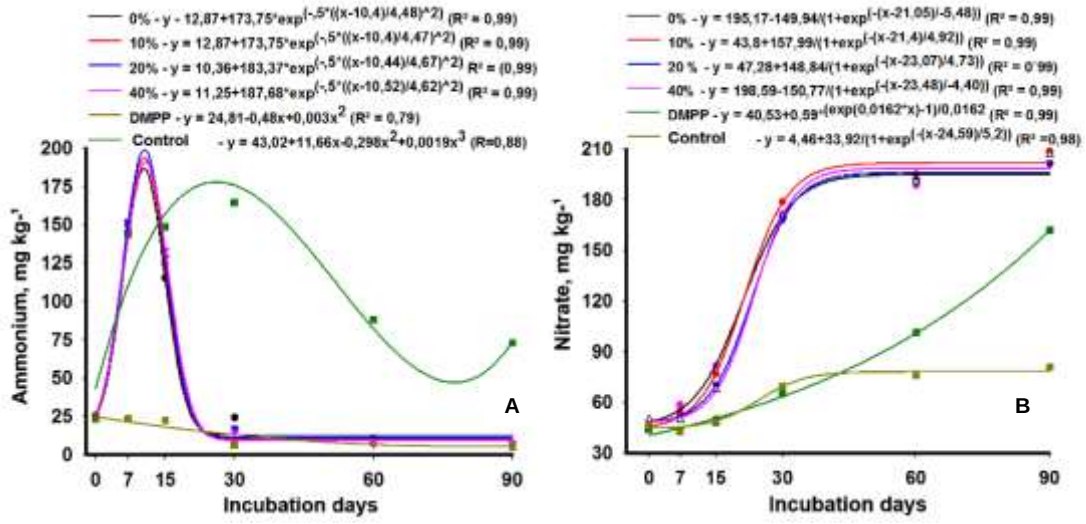

Fig. 3. Ammonium (A) and nitrate (B) concentrations in the soil in function of doses and sources of neem used to cover urea during the 90 days of incubation.

Discussion

The fact that ammonium levels increased in function of higher doses of neem covering urea (**15 days of incubation, Figure 1**) happened due to nitrification inhibition. When nitrification is delayed, higher concentrations of ammonium are observed in the soil.

Kumar *et al.* (2007) verified that nitrification inhibition varied from 4% to 30.9% when neem oil covered urea. Authors reported that there was direct influence of the oil meliacine levels on the nitrification percentage. Azadirachtin is a kind of meliacine and is the main component extracted from neem – every tissue of this plant may contain it (NAKATANI *et al.*, 1995; SIMÕES *et al.*, 2000).

In Gnanavelrajah's study (2001), neem leaf or seed powder efficiently inhibits nitrification by 54% and 48% respectively on the first week of analysis and by 24% and 19% on the fourth week. In the present study, there was proven inhibition up to 15 days of incubation due to the increase of neem doses. According to Gopal *et al.* (2007), their studies showed that azadirachtin suppressed the microbial community in the soil, also up to 15 days after incubation.

As equally observed in this experiment with neem cake and leaves powder compared to DMPP, Kumar *et al.* (2007) obtained positive results by using neem oil, but such results were inferior to another known molecule, nitrapyrin. On the other hand, Abbasi, Hina & Tahir's work (2011) observed better efficiency in nitrification inhibition (54%) using neem cake than the present study did up to 50 days of incubation.

It is important to say that, despite DMPP's efficiency in inhibiting nitrification under this laboratory experiment, no substantial difference was noticed regarding Tifton 85 growth and productivity on field with DMPP treatments when compared to common urea, as reported in experiment 1.

Barth, Von Tucher & Schmidhalter (2001) reported that DMPP's efficiency depends on soil texture. They went on to verify a strong correlation

between the soil's level of clay and DMPP absorption – the higher the absorption, the higher is the formation of nitrite (the first process in the conversion of ammonium to nitrate); in other words, the higher the levels of clay are, the higher DMPP's absorption is (and, because it is well absorbed, its efficiency decreases). In this laboratory experiment, a soil with medium texture was used, while, in the field experiment, it was a clayish soil. This particularity might have affected the product's efficiency under laboratory conditions. Nalin de Paulo (2012) noticed a higher DMPP's efficiency in inhibiting nitrification on quartzarenic neosol when compared to red-yellow latosol and red latosol. Furthermore, temperature in tropical regions might contribute to nitrification inhibitors inefficiency. According to Pasda, Hähndel & Zerulla (2001), high temperatures tend to accelerate DMPP's degradation, compromising its effectiveness.

Conclusion

Using neem leaves or cake to cover urea promotes inhibition up to 15 days after applying fertilizers. DMPP, however, is more efficient than neem when it comes to inhibit nitrification.

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