Plants adaptation to control nitrification process in tropical region; case study with *Acrocomia totai* and *Brachiaria humidicola* plants

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Abstract: In this study we focused on nitrification inhibition properties from tropical and subtropical plants; *Acrocomia totai* (palm tree) and *Brachiaria humidicola* (grass plant). Hexane extracted seed oil as well as dry powder of seed covers was applied in a quick nitrification bioassay for 24 or 50 hours to investigate their effects on nitrification process. Similarly *B. humidicola* shoot homogenates were applied in the same quick nitrification bioassay for their potential inhibitory effects on nitrification. Results showed that seed oil as well as seed covers from *A. totai* were significantly inhibited the nitrification rates in the quick nitrification bioassay test compared to distilled water control. In a separate experiment, it was found that shoots but not roots homogenates from *B. humidicola* grasses were efficiently retarded nitrification rates compared to water control. Therefore, including these plant genotypes together with main crops (intercropping) or as alternative crops, could effectively reduce soil nitrification process and leading to better N utilization.

Keywords: *Acrocomia totai*, nitrification inhibition, seed oil, *Brachiaria humidicola*, environment, plant nutrition

1 Introduction

Plants can take up different forms of nitrogenous compounds through their roots as well as their foliage. Ammonium has a positive charge and is generally retained by various soil colloids [8], so ammonium forms of nitrogen are more persistent in soil and give greater efficiency in terms of agricultural production [8, 15, 21]. The nitrification process means the microbial oxidation of the ammonium form of nitrogen to nitrite and then to nitrate by *Nitrosomonas* and *Nitrobacter* groups of bacteria, respectively [16, 17]. This process very actively participates in environmental pollution by different nitrogenous gasses and underground water nitrate contamination, particularly in tropical and subtropical agroecosystems. In addition, it has an active role in land degradation by disturbance of either the aquatic biosystem, soil salinity or soil acidification [2, 5]. It has been known for several decades, that natural stands of grass have the ability to control nitrification rates in the soil [6, 20]. The inhibition of nitrification by plant substances could be an adaptation of those plants to low pH and soil acidification induced by the nitrification process or by climatic conditions [15]. This inhibitory effect has potential advantages for agricultural and environmental applications [18, 24]. Thus although several natural substances have been evolved to retard nitrification rates in agricultural systems, there is still great potential for detection of new products with high biological activity and different modes of action [12]. Natural or synthetic nitrification inhibitors act by blocking the enzymatic activity pathways of bacteria which are responsible for ammonium oxidation. Nowadays, various commercial nitrification inhibitors exist on the market. In some cases they are incorporated into N fertilizers as is the case for ENTEC; which is a nitrification inhibitor comprising ammonium sulphate plus DMPP (3,4-Dimethylpyrazole phosphate). ENTEC helps to slow down nitrification rates and to guarantee ammonium nutrition of plants in the soil. Despite getting influenced by various climatic factors, there are reports of retardation of the nitrification process by 3-7 weeks [14, 15, 24] due to application of various nitrification inhibitors. Nevertheless, application of such chemicals could be dangerous to both the environment and the biosphere and it’s likely their use will be limited in the future. Synthetic nitrification inhibitors such as DMPP or DCD (dicyandiamide) are also expensive and not
available for all farmers around the world [15]. From an ecological perspective, natural products represent more suitable alternatives [17]. Various plant-based substances such as those derived from parts of Azadirachta indica trees [5, 13, 20] have been found to have biological activity in reducing nitrification rates in soil and in improving nitrogen efficiency in agricultural systems. In addition, plant polyphenols, terpenes, essential oils and different types of quinines [4, 9, 11] as well as fatty acids [21, 22] have been found to retard nitrification rates in the soil. On the other hand, in contrast to synthetic acids [21, 22] have been found to retard nitrification rates and different types of quinines [4, 9, 11] as well as fatty acids [21, 22] have been found to retard nitrification rates in the soil. Therefore, nitrification inhibitory properties of plant materials possessing properties of nitrification inhibition are safer and offer potential benefits to agriculture and the environment. Brachiaria plants are C₄ species and among the most important pasture crop widely adapted to grow in tropical and subtropical parts of South America, Africa and Asia [1]. For example, different Brachiaria species account for 85% of total planted pasture area in South America [10], and they are also commercially very important in the tropics, particularly in Brazil. These plants are well adapted to ammonium nutrition, more so than any other crop [16]. Focus has recently turned to B. humidicola for its potential in production and release of natural nitrification inhibitors [16, 17, 19, 21]. However, there are no reports regarding the nitrification inhibitory of Acrocomia species in the literature. The genus Acrocomia belongs to palms (Arecaceae family) which grow as wild tropical trees in South America. Oils from different parts of Acrocomia seed are used in soap, washing powder, pharmacy and food products. This palm tree prefers more temperate environments than other American palms [7]. During Earlier soil testing observations by the author it was observed that soils collected under stands of this palm in Paraguay, had low nitrate content. This has led to the present study in which the nitrification inhibition of Acrocomia and Brachiaria plants as well as various concentrations of linoleic acid have been investigated under laboratory conditions.

2 Material and Methods

2.1 Plant materials

Seeds from A. totai were supplied from a commercial stand in Paraguay. Seeds had a very hard covering which was first removed using hammer and a special saw to expose the nut. Nut (or endosperm) oil was extracted using hexane (1:3 w/v) for 3 minutes, after washing and cleaning the nuts. The extract was then separated from hexane by vacuum filtration in a rotary evaporator at 35°C and 100-30 mb pressure.

In another experiment, seeds of B. humidicola (Rendle) grasses (accession 26159, obtained from Dr Volker Roemheld, CIAT, Colombia) were germinated at 25°C in fine sands (0.02-0.5 mm) under controlled conditions. Seedlings then were transferred into nutrient solution in a growth chamber under controlled conditions (day/night temperature of 28/25±2°C, relative humidity of 65% and 300-350 µmol cm⁻²s⁻¹ light intensity). The composition of nutrient solution was 10 µM H₃BO₃, 0.5 µM MnSO₄, 0.5 µM ZnSO₄, 0.1 µM CuSO₄, 0.01 µM Mo₇O₂₄(NH₄)₆, 83 µM Fe-EDTA, 0.7 mM K₂SO₄, 0.5 mM KH₂PO₄, 1.2 mM MgSO₄, 1.2mM KCl. For ammonium treatments 1 mM CaCl₂ was used as the calcium source [15]. Nitrogen in the form of either ammonium or nitrate, was added into the nutrient solution. Plants were grown for several weeks, after which root and shoot homogenates were prepared by grinding fresh root and shoot materials in liquid nitrogen. Middle leaves on the main stem were used for homogenate preparation.

2.2 Incubation experiments

In this study, different incubation experiments, at room temperature of 20±3°C, were performed:

a) Various amounts of variables such as root or shoot homogenates, seed oil, powdered seed cover, or different concentrations of linoleic acid were included in a bioassay test to determine their effects on the nitrification process. An aliquot of 20 µl of the A. totai seed oil was added directly (without any other interfering solvents) to each sample in a rapid nitrification potential detection test (bioassay) for either 24 or 50 hours incubation.

b) Various amounts of powder of A. totai seed cover (10, 20 and 30 mg per sample) were included in the incubation experiment.

c) 0.25 or 0.50 g of fresh root or shoot homogenates from B. humidicola were applied to soil in a quick bioassay test to determine their potential effect on the nitrification process.

d) Linoleic acid at concentrations of 0.05, 0.1, 0.2 and 0.5 percent was applied in the soil bioassay incubation test to determine its potential effect on the nitrification process.
2.3 Soil Bioassay

A soil bioassay for rapid determination of nitrification inhibitory potential of *Acrocomia* seed oil and *Brachiaria* root and shoot homogenates was adopted following Kandeler, [3] with some modifications. The adopted procedure included 2.5 g of fresh activated soil [15], 7.5 ml of (NH₄)₂SO₄ 13.3 mM, 60 µl of NaClO 1.5 M, 2.5 ml distilled water ± *Acrocomia* seed oil (20 µl), or *Brachiaria* shoot and root homogenates (0.25 or 0.5 g), or DMPP (3,4-Dimethylpyrazole phosphate) or distilled water. Each of these was transferred into 50 ml plastic bottles containing 2.5 g of a fresh activated standard soil. DMPP was used as a standard nitrification inhibitor in a concentration of 10% of N-NH₄⁺ to give more informative results. Bottles were shaken for 24 h at 200 rpm and then extracted using 7.5 ml of 2M KCl, then filtered and measured colorimetrically at 540 nm following Kandeler [3]. Sulfanilic acid was used as the reagent to produce a rose colour if nitrite was present, with less colour development signifying inhibition of nitrification by the test substance.

Excel software was used to draw the figures and SPSS software was used for analysis of data. Comparison of means was performed at P= 0.05 using Duncan’s multiple range test. Data in figures are presented as average of four replicates ± SD.

3 Results

In the present study, the amount of nitrite produced was equal to nitrification strength. When hexane extracted seed oil of *A. totai* (20 µl) was used in our 24h bioassay, it showed significant ability to inhibit nitrification compared to water control (Fig. 1). The degree of inhibition was similar to that induced by DMPP application. Water controls, which comprised only water and ammonium sulphate, produced the most nitrite (Fig. 1). When different amounts of powdered acrocomia seed cover were bioassayed, nitrification was inhibited compared to control (Fig. 2). All three levels of 10, 20 and 30 mg of applied seed cover showed significant nitrification inhibition compared to water control. There was positive correlation between nitrification inhibition rates and seed cover concentration. Nevertheless, DMPP maximally inhibited nitrification (Fig. 2).

When 250 and 500 mg of fresh shoot homogenates, and 500 mg of fresh root homogenates were applied in the bioassay test (Fig. 3), it was shown that 500mg *Brachiaria* shoot homogenate was more effective on inhibition of soil nitrification than 250 mg. Both doses of shoot homogenate significantly reduced nitrification rates compared to water control; with a stronger effect in the 500mg sample (Fig. 3). The inhibitory effect of 500 mg shoot homogenates was also significantly stronger than standard DMPP nitrification inhibitor. On the other hand, application of 500 mg fresh root homogenates had no significant effect on nitrification rate in the bioassay (Fig. 3).

When shoot homogenates of ammonium or nitrate or ammonium-nitrate fed plants were assayed, shoot homogenates of *B. humidicola* significantly inhibited nitrification rates during 50 h incubation period, independent of nitrogen form (Fig. 4). There was no significant difference among ammonium, nitrate or ammonium nitrate grown plants regarding their inhibitory effect on nitrification rates. (Fig. 4).

In the last experiment (Fig. 5), different concentrations of linoleic acid were applied for 24 h in the soil bioassay test to detect their potential nitrification inhibition as Subbarao et al. [17] proposed. Linoleic acid significantly reduced nitrification rates compared to d-water control (Fig 5). Linoleic acid concentrations of 0.2% (20 µl) and 0.5% (50 µl) inhibited nitrification more efficiently than lower concentrations of (0.05% and 0.1%). At 0.2% and 0.5% Linoleic acid inhibited nitrification more strongly than DMPP.

When *Brachiaria* shoot homogenates were incorporated into the soil and incubated for 4 weeks (Fig 6), there was significant inhibition of nitrification compared to control. However, there was no significant difference between DMPP and the shoot homogenates until after the fourth week.

4 Discussion

In the present study, application of seed oil and seed cover from *A. totai* as well as shoot homogenates from *B. humidicola* significantly reduced soil nitrification rates in the bioassay (Fig 1-5). Such effects were also reported by other studies [6, 11, 20, 21]. However, in these studies referred to root exudates not plant materials [21, 22]. It has in fact been known for several decades that plants have the ability to limit the degree of soil nitrification [9]. Plants can manipulate their biochemical, physiological and morphological characteristics in response to environmental factors variations. The extent of such changes usually determines the ability of a species to succeed under temporary or permanent environmental stress. In the present study, various plant materials of the two plant species were able to reduce nitrification rates. These two plant species are native to South American
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Fig. 1. Amount of nitrite produced under application of 20 µl seed oil (from A. totai) compared to water control and DMPP control (10% of N-NH₄⁺) in 24 h incubation test. Each sample consisted of 2.5 g fresh soil+7.5 ml of 13.3 mM (NH₄)₂SO₄+50µl NaClO₃+2.5 ml d-water ± distilled water or DMPP or seed oil. Comparison of means was done at 5% of Duncan test. Data are the averages of four replicates ± SD

Fig. 2. Mean value of nitrite produced with application of different amounts of seed cover (from A. totai) during 24 h incubation test. Each sample consisted of 2.5 g fresh soil+7.5 ml of 13.3 mM (NH₄)₂SO₄+50µl NaClO₃+2.5 ml d-water ± X mg of powder from seed cover (10, 20 30 mg). Comparison of means was done at 5% of Duncan test. Data are the averages of four replicates ± SD

Fig. 3. Mean value of nitrite produced by application of 0.250 or 0.500 g shoot homogenate and 0.500 g root homogenates from B. humidicola during 50 hour incubation period. DMPP was used with a concentration of 10 times of normal concentration (1% of N-NH₄⁺). Comparison of means was done at 5% of Duncan test. Data are averages of four replicates ± SD
Fig. 4. Mean value of nitrite produced by application of 0.5 g fresh shoot homogenates from *B. humidicola* during 50 hour incubation period. Plants were grown with 2 mM N as ammonium sulphate, calcium nitrate, or ammonium nitrate. DMPP was used with a concentration of 10 times of normal concentration (1% of N-NH₄). Data are averages of four replicates ± SD.

Fig. 5. Mean value of nitrite produced with different concentrations of linoleic acid compared to water control and DMPP control (10% of N-NH₄⁺) in a 24 h incubation period. Linoleic acid was used in 0.05, 0.1, 0.2 and 0.5 percent equals to 5, 10, 20 and 50 µl per sample. Comparison of means was done at 5% of Duncan test. Data are the averages of four replicates ± SD.

Fig. 6. Effects of incorporation of shoot homogenates from *B. humidicola* (one g/kg dry soil) on soil nitrification rates during four weeks incubation under laboratory room temperature of 20±2°C. Ammonium sulfate was added onto the soil based on 150 mg N-NH₄⁺. In Brachiaria treatment ammonium sulfate was added onto the homogenated powder and then mixed uniformly with the soil. Small plastic pots containing 200 g dry soil was used for this experiment. Comparison of means was done at 5% of Duncan test.
countries and are thus adapted to a stressful environment, where the soil has high nitrification rates and acidic conditions. It is quite important that parallel interactions between stress factors in these low-fertility acid soils must always be considered [23]. Thus nitrification as well as denitrification processes result in limited N status of soil for a plant’s root uptake. Therefore, ability of plant tissues to inhibit nitrification could be a common and natural response to those stressful conditions. In supporting these findings, we have shown that the mechanism of release of nitrification inhibitory compounds in root exudates of B. humidicola is probably not an active process [17] as supposed by Subbarao et al. [21]. In the present study, root homogenates did not show significant inhibition of nitrification (Fig. 3). So, to make use of the natural inhibitory effects of Brachiaria plant, their shoots need to be incorporated into the soil very regularly, as in the last experiment where one g Brachiaria shoot homogenate was added per kg soil (Fig. 6), until the end of the second week, whereupon the rate of nitrification inhibition of Brachiaria shoot homogenates was similar to DMPP. Any difference in efficiency of nitrification inhibition between DMPP and Brachiaria shoot homogenates only emerged after 4 weeks.

In the next incubation experiment (Fig. 5) application of different concentrations of Linoleic acid was able to inhibit nitrification at all concentrations tested (Fig 5) and at concentrations of 0.2% and 0.5% this effect was even more effective than DMPP, the most effective and widely used nitrification inhibitor in the world. This indicates that in Brachiaria shoot or seed cover of Acrocomia, besides Acrocomia seed oil, fatty acids could be candidate substances for inhibition of nitrification.

The role of unsaturated oil [21] and essential oils [11] from various plant species, on inhibition of soil nitrification has been previously demonstrated (11). In addition, the seed oil from A. totai was also very effective on inhibition of nitrification; however, most of the oil consisted of saturated fatty acids (71.24%, with mono unsaturated fatty acids at 25.75% and poly unsaturated fatty acids: 3.01%). Nevertheless, minute amounts of unsaturated fatty acids are enough to exert strong inhibition on soil nitrification as was shown in Fig. 5. So, the inhibitory effect of Acrocomia seed oil is probably due to its unsaturated fatty acids component, while in seed cover as well as in shoot homogenate of Brachiaria plants other compounds may also be involved.

Such Inhibition of nitrification by these plant materials suggests that fatty acids might be the active constituent as recently suggested by Subbarao et al. [21,22] who showed that fatty acids from B. humidicola could have significant nitrification inhibitory effect due to its content of unsaturated fatty acids. Similarly, in this study we showed that applied linoleic acid has strong inhibiting effects on nitrification rates (Fig. 5). Unsaturated fatty acids (in oil or in shoot homogenates or in seed cover) seems likely to be the active ingredient. Many plants contain oils, fats or waxes and various other bioactive compounds associated with their defence mechanisms against pathogens as well as against unfavourable environmental conditions. Various natural branched fatty acids possess fungistatic and bacteriostatic properties. In a larger perspective, this could be seen as an adaptive mechanism of natural stands against soil acidification and nutrient loss through nitrification. Nevertheless, for ecological production of human foods, these natural alternatives could replace synthetic nitrification inhibitors, or at least be incorporated into cultivation systems by intercropping.

In various incubation experiments, the inhibitory effect of 500 mg shoot homogenates from B. humidicola was also significantly stronger than standard DMPP nitrification inhibitor. This in part, beside the proposed fatty acid effect, likely to be due to the fixing properties of various bioactive substances in shoot homogenates which could act as unspecific nitrification inhibitors.

5 Conclusion

In conclusion the results showed that various materials of these two plant species showed promise as inhibitors of nitrification. Seed oil and seed cover from A. totai, as well as shoots but not root homogenates from B. humidicola also strongly suppressed the microbial nitrification process in the soil. This study and others has shown that, various fatty acids, particularly unsaturated fatty acids, to be the the most likely source of these inhibitory effects on nitrification.

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