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Short Communication

# Impact of azadirachtin, an insecticidal allelochemical from neem on soil microflora, enzyme and respiratory activities

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**Abstract**

The effect of 10% azadirachtin granules (alcoholic extract of neem seed kernel mixed with China clay) was studied on the population of bacteria, actinomycetes, fungi, *Azotobacter* and nitrifying bacteria; soil dehydrogenase, phosphatase and respiratory activities on 0, 15th, 30th, 60th and 90th days after application in sandy loam soil collected from the fields. It was observed that baring the *Azotobacter* sp., azadirachtin at all the doses exerted a suppressive effect on the rest of the microbial communities and enzyme activities in the initial 15 day period. The population of bacteria, actinomycetes besides phosphatase and respiratory activities recovered after 60th day and subsequently increased significantly. The fungi and nitrifiers were most sensitive groups as their numbers were reduced significantly throughout the studies. The two times and five times recommended dose of azadirachtin had very high biocidal effects on the soil microorganisms and its activities. However, analysis of the data by the Shannon Weaver index showed that azadirachtin reduces both the form and functional microbial diversity at all doses.


*Keywords:* Azadirachtin; Enzymes; Microflora; Neem; Soil

## 1. Introduction

Neem tree (*Azadirachta indica* Juss), a member of the Meliaceae family, grows commonly in tropical areas of Asia, particularly India. More than 10 limnoid allelochemicals have been isolated from various parts of the neem tree, belonging to nine basic structure groups *viz.* azadiron (from oil), amoorastatin (from fresh leaves), vepinin (from seed oil), vilasinin (from green leaves), gedunins (from seed oil and bark), nimbin (from leaves and seed), nimbolin (from kernels), salanin (from fresh leaves and seed) and azadirachtin (from neem seed) (Kraus, 2002). Of all the compounds isolated from the plant, azadirachtin, a highly oxygenated C-secomeliacin is the most toxic alkaloid (Ascher, 1981).

Azadirachtin possesses insecticidal activity against many economically important insect pests such as *Helicoverpa armigera*, *Spodoptera litura*, *Plutella xylostella*, *Sitophilus oryzae*, *Sitophilus zeamis*, *Earis vitella*, *Aphis gossypii*, *Bemicia tabaci*, *Pectiniphora gossypiella*, nematodes like *Cosmopilitis sordidus* etc. (Schmutterer and Singh, 2002). Currently, there is an upswing in the use of azadirachtin as an insecticide owing to its plant origin. The belief that such natural insecticides are safe or less damaging to the ecosystem is also necessary to be further validated, as their effect on non-target organisms like crustaceans (*Daphnia magna* and *H. Aztec*) have been reported to be very close to threshold chronic toxicity (Scott and Kaushik, 1998). A low short-term toxicity of azadirachtin, neem extracts and their products Eneem 3 G and Neemix 4.5 E on the mycorrhiza *Glomus intraradices* has been described (Wan and Rahe, 1998). It is well known that indiscriminate methods of application and concentrations of insecticides are rampant, often allowing high loads of the xenobiotics to reach the soil matrix.

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The aim of this work was to study the effect of azadirachtin granules (10% *a.i.*) on the population and diversity of the important soil microbiota, enzyme and respiratory activities.

## 2. Methods

The soil was collected randomly from different parts of the field of the IARI farm from a 0 to 6 in. depth. It was homogenized, shade dried and sieved through a 2 mm sieve. The soil was sandy loam with the following composition (%) – 0.253 carbon, 0.03 total nitrogen and 0.003 available phosphorus. The pH was 7.4 and soil water ratio 1:2. Seeds were harvested from neem trees in the vicinity of the IARI that had already been identified to contain more than 1000–1250 ppm of azadirachtin. The seeds were air dried and azadirachtin was extracted using methanol as the organic extractant. Azadirachtin thus extracted was mixed with China clay to produce 10% *a.i.* azadirachtin granules. In-house studies established that the recommended dose of azadirachtin granules for soil application was 20 kg $ha^{-1}$.

For the present study, 5 kg of soil was transferred to pots (14 in. × 10 in.). Four treatments were included with the application of azadirachtin granules at recommended dose (1×), twice (2×) and five times (5×) the recommended dose and a control without any application to the soil. Each treatment had three replicates. The soil was brought to 33% moisture level by adding sterile water to each pot. The whole composition of soil, azadirachtin granules and water was mixed thoroughly and covered with filter paper and incubated at room temperature (ranging from 28 to 32 °C). Throughout the incubation period, the soil was kept at optimal moisture condition. Soil samples were taken at 0, 15th, 30th, 60th and 90th day and the following microbial studies were carried out.

The enumeration of the soil microflora was done by the dilution plate method (Nair and Subba Rao, 1977). The total colony forming units (cfu) of bacteria, fungi, actinomycetes and free-living $N_2$-fixers were recorded on nutrient agar (Allen, 1959), Ken Knight and Munaier's agar (Allen, 1959), Martin's rose bengal agar (Martin, 1950) and Jensen's agar (Jensen, 1951) media, respectively. The plates were incubated at $28 \pm 2$ °C and microbial population was calculated and expressed as cfu $\times 10^n$ $g^{-1}$ air dried soil, where $10^n$ was dilution factor. The population of bacteria involved in nitrification process was determined by the most probable number method (MPN) (Alexander and Clark, 1965). The activity of dehydrogenase enzyme was estimated spectrophotometrically at 485 nm through the production of triphenyl formazan from triphenyl tetrazolium chloride (Klein et al., 1971) and phosphatase activity was assayed by estimation of *p*-nitrophenol (at 400 nm) released by phosphatase activity when soil was incubated with buffered (pH 6.5) sodium *p*-nitrophenyl phosphate (Tabatabai and Bremner, 1969). The respiratory activity of the soil sample under different treatments was measured manometrically using Warburg's constant volume respirometer, which is one of the most reliable methods for this study. It is based on the principle that at constant temperature and constant gas volume, any changes in the amount of gas can be measured by changes in its pressure (Umbreit et al., 1972).

The results were statistically assessed through analysis of variance (ANOVA) for two-way classification (Gomez and Gomez, 1984). Besides the individual microbial community response to azadirachtin, the response at microbial biodiversity level was assessed employing the Shannon Weaver Index ($H^1$) by the following equation (Shannon and Weaver, 1949)

$$H^1 = -\sum_{i=1}^{S} p_i \ln p_i$$

where $H^1$ is the Shannon Weaver Index, $p_i$ is the proportion of the individual species to total and $S$ the number of communities.

The $H^1$ values are divided by $Log_2$ to normalize the data obtained from three groups.

## 3. Results and discussion

Azadirachtin granules at recommended dose suppressed the bacterial population in the initial 15 days, and later had an enhancing effect with maximum count reaching $57 \times 10^6$ cfu/g soil by the 90th day, where as higher doses had a negative impact compared to the normal dose, which was however, on par with control (data not shown). The actinomycetes population also responded similarly with initial dip in the numbers up to 15 days in the treatment when recommended dose was applied and increased significantly after 60 days incubation. The five times recommended dose (5×) significantly suppressed the actinomycetes number through out the study with lowest count of $22 \times 10^3$ cfu encountered on 90th day (data not shown). Fungal community was highly sensitive to all three doses of azadirachtin through out the incubation studies, with lowest population of $10 \times 10^3$ cfu recorded on 60th day at five times recommended dose (data not shown). In the control treatment, it was observed that population of certain microbial communities, like bacteria and actinomycetes, decreased significantly during the 60th day of observation followed by a sharp increment in the 90th day of observation. This reflects the classic growth curve pattern exhibited by microorganisms. Increase in population is the resultant of free availability of nutrients followed by production of toxic metabolites by the burgeoning populations, which causes death of the individual cells resulting in a decline in the numbers of microflora. Release of nutrients by the dead cells stimulates the growth again. However, if an increase or a decrease in microbial population is not very intense and significant, the subsequent response in their numbers is also vice versa. Whereas, in azadirachtin amended soils, the dynamics of microbial population is

determined by the interactions of the allelochemical with the microorganisms.

Much of the work reported on the effect of azadirachtin on soil microbial population and its activities is in terms of amendment of soil with neem oil cake or neem seed cake, which contains only trace amounts of azadirachtin alkaloid. Additions of organic amendments have improved the bacterial population (Mukherjee et al., 1991; Gopal et al., 2001). However, we observed that application of azadirachtin (extracted from neem seed kernel) granules significantly reduced the bacterial and fungal populations up to a 15 day period. Many workers have earlier confirmed the anti-microbial property of azadirachtin (Coventry and Allan, 2001), besides anti-fungal activity too (Govindachari et al., 2000).

Among the function specific microbial communities, the free living nitrogen fixer *Azotobacter* was observed to proliferate to very large number in the soil treated with the recommended dose of azadirachtin, with the highest population of $107 \times 10^2$ cfu/g soil achieved by 90th day, while other doses had similar effect as control (data not shown). In this aspect, the effect of azadirachtin was parallel with that of neem oil cake, i.e. in stimulating the numbers of diazotroph *Azotobacter* (Pandey and Singh, 1990; Gopal et al., 2001). The chemoautotrophic nitrifying bacteria *Nitrosomonas* and *Nitrobacter* were strongly suppressed by the azadirachtin at all the doses, with five times being extremely detrimental (data not shown). The use of azadirachtin to coat urea is a common practice as it reduces the loss of nitrogen by preventing the activity of nitrifiers. Neem, as an inhibitor of nitrifiers, has been well documented (Usha Kiran and Patra, 2003) and the present observation also supported this. Although the capacity to suppress the nitrifiers' activity by azadirachtin is seen as an advantageous point in crops requiring ammoniacal nitrogen, accumulation of $NH_4^+$ ions in the soil in a natural ecosystem is considered to be toxic. In this aspect, azadirachtin could become the culprit for preventing nitrification in the soils, which naturally have higher $NH_4^+$ concentrations. However, at the same time, it becomes beneficial in soils where the $NH_4^+$ ion concentration is high and crops do not get a steady and sustainable source of $NO_3^{2-}$ nitrogen nutrition.

The soil dehydrogenase activity was not influenced either negatively or synergistically by all the three dosages of azadirachtin granules. However, the recommended dose had increased this enzyme activity (Table 1). The phosphatase activity was significantly boosted by the recommended dose of the botanical pesticide, whereas the five times dosage heavily curbed this enzyme activity (Table 1). The overall activity of these enzymes decreased gradually from the initial sampling period to the final one.

It was observed that at the recommended dose of azadirachtin granules, this enzyme activity was on par with the control, though the same dose had significantly increased bacterial, actinomycetes and *Azotobacter* population. The

Table 1
Impact of azadirachtin granules (AG) on soil dehydrogenase [**A**], phosphatase [**B**] and respiratory activity [**C**]

| Treatments | Days of observation | | | | Mean |
|---|---|---|---|---|---|
| | 15 | 30 | 60 | 90 | |
| **[A]**[a] | | | | | |
| Control | 38.00 | 31.00 | 28.00 | 1.00 | 29.50 |
| AG – 1× | 41.00 | 36.00 | 31.00 | 29.00 | 34.25 |
| AG – 2× | 39.00 | 24.00 | 28.00 | 23.00 | 28.50 |
| AG – 5× | 38.00 | 21.00 | 28.00 | 25.00 | 28.50 |
| Mean | 39.00 | 28.00 | 28.75 | 24.50 | – |
| CD ($p = 0.01$) | Treatments NS | Days 5.433 | | Treatment × days NS | |
| **[B]**[b] | | | | | |
| Control | 21.00 | 13.00 | 12.00 | 08.00 | 13.50 |
| AG – 1× | 20.00 | 22.00 | 20.00 | 17.00 | 19.75 |
| AG – 2× | 20.00 | 18.00 | 14.00 | 10.00 | 15.50 |
| AG – 5× | 16.00 | 08.00 | 08.00 | 06.00 | 09.50 |
| Mean | 19.25 | 15.25 | 13.50 | 10.25 | – |
| CD ($p = 0.01$) | Treatments 3.397 | Days 3.397 | | Treatment × days NS | |
| **[C]**[c] | | | | | |
| Control | 12.00 | 09.30 | 11.00 | 11.00 | 10.82 |
| AG – 1× | 10.00 | 10.60 | 13.60 | 14.00 | 12.05 |
| AG – 2× | 11.30 | 12.00 | 10.00 | 09.60 | 10.75 |
| AG – 5× | 10.30 | 07.30 | 06.60 | 06.30 | 07.62 |
| Mean | 10.90 | 09.80 | 10.30 | 10.22 | – |
| CD ($p = 0.01$) | Treatments 1.773 | Days NS | | Treatment × days NS | |

(Results are an average of nine replicates.)

[a] Initial activity: 37.40 mg TPF $\times 10^{-4}$ $h^{-1}$ $g^{-1}$ of soil.

[b] Initial activity: 23.12 μg of *p*-nitrophenol $h^{-1}$ $g^{-1}$ of soil.

[c] Initial activity: 38.16 μl $O_2$ uptake $h^{-1}$ $g^{-1}$ of soil.

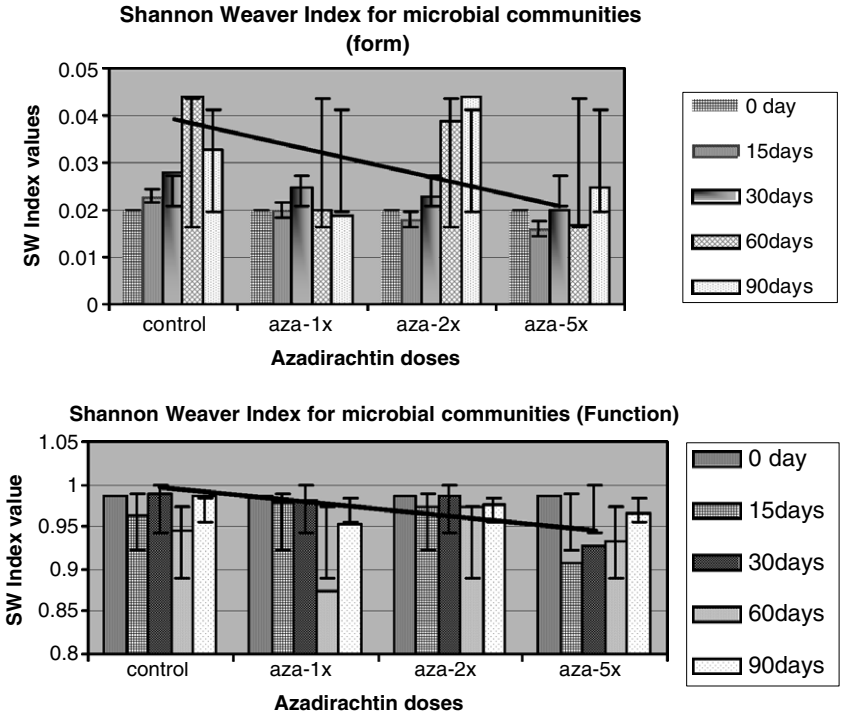


Fig. 1. Transformation of microbial data to the SW index for evaluating the effect of azadirachtin granules on microbial diversity at the form and functional level.

reason could be that this increase was nullified by the decrease in the fungal and nitrifiers' population. The biocidal effect of azadirachtin on the soil microorganisms and subsequent decomposition and release of the phosphates from the dead microbial biomass could have acted as a resource for high phosphatase activity. Antonious (2003) reported that an azadirachtin based insecticide Neemix-4E application had some transitional effects on urease enzyme activity, which was neither drastic nor prolonged enough to be considered deleterious to the soil microorganisms and their activities important in soil fertility.

The respiratory activity of the soil microflora also responded similar to that of the phosphatase enzyme, with the recommended dose of the azadirachtin granules significantly improving this metabolic activity and the five times dosage suppressing it. The intermediate dose was seen to be neutral (Table 1). The increase in respiratory activity at the recommended dose could be attributed to the increase in bacterial populations feeding on carbon sources deposited due to death of the other microbial communities killed by the allelochemical, which were active metabolizers. An increased respiratory activity was reported similarly by Devakumar and Mukherji (pers. commun.).

The Statistical approach of Shannon's index was also employed for understanding the effect of azadirachtin at the community level i.e. on form (bacteria, fungi and actinomycetes) and function-specific (free-living nitrogen fixers and nitrifiers) microbial communities (Fig. 1). It was observed that the general communities reduced at all doses and up to a period of 30, days we got a linear response in the suppression, whereas in the control treatment the richness of bacteria, fungi and actinomycetes were noticed at all times. In the case of function specific communities, the recommended dose, twice and five times recommended dosage of azadirachtin granules proved to have a decreasing effect on diversity in most cases of observations. This clearly showed that the overall effect of azadirachtin on form and functional community level reflected that of the individual effect too.

A salient feature observed in this study was that neem "actives" started asserting their suppressive nature on the fungi and the nitrifiers within the first month of incubation. However, an improvement in the population of bacteria, actinomycetes, *Azotobacter* and enzyme and respiratory activities was noted only around the 60th day and afterwards. Hence, it could be presumed that once some of the microbial population was suppressed the competition for the nutrition eases and allowed the other microflora to proliferate. The whole gamut of influence of the neem "actives" on the microflora was observed conspicuously within a two month period even though the $DT_{50}$ (time taken for 50% disappearance of the initial concentration) for the azadirachtin A and B was observed to be 20 days at 25 °C (Stark and Walter, 1995) and depended upon organic matter content and temperature of the soil (Agyarko et al., 2006).

## 4. Conclusion

From this study, it was clear that application of such chemically derived neem pesticides, particularly azadirachtin, at higher than the recommended doses could become

counterproductive to soil fertility. The natural and highly organic neem oil cake is a better alternative to this, as it acts as a pesticide and at the same time provides the much necessary nutrition to the soil microbes, besides improving the soil physico-chemical properties. However, in order to judge the overall long-term effects of inputs of azadirachtin on soil microbial biodiversity, extensive work should be done in different cropping systems and soil varieties with a specific agriculturally important group of microorganisms for achieving a comprehensive understanding.

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