

Influence of Physicochemical Parameters of Neem (*Azadirachta indica* A Juss) Oils on Nitrification Inhibition in Soil

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The technology for the production of neem oil coated urea (NOCU) developed by the Indian Agricultural Research Institute is in the pipeline for adaption by several Indian fertilizer industries. Use of nitrification inhibitors is one of the methods of improving the nitrogen use efficiency (NUE) of nitrogenous fertilizers in agriculture. However, standard specifications for the neem oil as a raw material of NOCU are desired. Accordingly, the present study was undertaken to evaluate 25 samples of neem oils comprising 11 samples of expeller grade (EG) oils, 8 samples of cold-pressed (CP) oils, 3 samples of solvent-extracted oils, and 2 commercial formulations. NOCU was prepared using these oils (5000 ppm of urea-N). The soils fertilized with NOCUs (200 ppm of urea-N) were incubated at 27 °C and 50% water-holding capacity for a period of 15 days. Nitrapyrin (0.5% of N) coated urea served as the reference and prilled urea as control. Samples were analyzed for NH₄⁺-N, NO₂⁻-N, and NO₃⁻-N using standard methods. The percent nitrification inhibition (NI) was calculated, and the results revealed that all of the neem oils caused NI ranging from 4.0 to 30.9%. Two samples of EG oils and two commercial formulations were found to be the best, causing 27.0–30.9% NI. Iodine, acid, and saponification values and meliacin content of all of the oils were analyzed and correlated with NI. The results revealed the direct influence of meliacin content of the neem oils on NI, which, however, was found to be negatively correlated with saponification and iodine values. There is, therefore, a need to introduce new Bureau of Indian Standards (BIS) specifications for neem oils as raw materials of NOCU.

KEYWORDS: Neem (*Azadirachta indica*); neem oils; nitrification inhibition; meliacins; NOCU; iodine value; saponification value; acid value

INTRODUCTION

The commonly used nitrogenous fertilizers such as urea suffer from low nitrogen use efficiency (NUE) and contribute to environmental pollution. Worldwide, the NUE for cereal production (wheat, corn, rice, barley, sorghum, millet, oat, rye, etc.) is approximately 33%. The unaccounted 67% represents a U.S. \$15.9 billion annual loss, assuming fertilizer–soil equilibrium (1). Excessive nitrification is one of the key factors of inefficient N use and consequent environmental hazards such as denitrification and nitrate leaching. The use of nitrification inhibitors minimizes these ill effects (2). The Indian Agricultural Research Institute pioneered the discovery and development of neem products as fertilizer urea adjuvants (3, 4). A field trial demonstration confirmed the efficacy of solvent extract of neem seeds as urea adjuvants (5). Devakumar (6) identified certain

key meliacins responsible for the nitrification retardation activity of neem products.

A new dimension was added to this study by the process development of neem oil coated urea (NOCU) (7). The potential of neem oil as an industrial commodity for coating on urea was first demonstrated by Suri (8) at the National Fertilizer Limited (NFL)'s plant in Panipat, Haryana (India). An industrial level demonstration of the production of NOCU using our patented technology (9) was carried out at two fertilizer plants, namely, KRIBHCO, Hazira (June 1999 and December 1999) and Sri Ram Fertilizers and Chemicals, Kota (December 2000), by producing over 1000 metric tonnes. M/s National Fertilizer Ltd., Panipat, has independently produced NOCU. The efficiency of NOCUs thus produced by both of the processes has been evaluated in farmers' rice fields and was found to give higher rice grain yields by 6.3–11.9% in all cases except at Bhatinda, Punjab (10, 11). Singh et al. (12) carried out evaluation of the NUE of various slow-release ureas in Nainital on wheat and found that NOCU compared favorably with sulfur and lac coated

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urea. Furthermore, it was also found that ammonia volatilization was the least with NOCU treatment (13).

As the demand for NOCUs is on the increase, more stringent specifications for neem oils have been sought by the Indian Council of Agricultural Research (ICAR) and the Ministry of Chemicals and Fertilizers, Government of India. Accordingly, the present study was undertaken to identify the most suitable industrial grades of neem oil and the influence of physicochemical properties of neem oils on nitrification inhibitory activity of NOCUs.

MATERIALS AND METHODS

Neem Oils. Twenty-five samples of neem oils having diverse backgrounds in terms of ecotype, method of preparation, age of the sample, etc., were procured from the market and different research institutions.

Test Soil. Sandy loam [Inceptisol] was collected from the research farm of the Institute. The physicochemical properties were as follows: sand, 60.8%; clay, 20.5%; silt, 18.7%; pH 8.4 (soil/water 1:2.5); EC at 25 °C, 0.35 dSm⁻¹; organic carbon, 0.26%; available N, 0.6 g kg⁻¹ of soil; nitrate-N, 16.3 mg kg⁻¹ of soil; nitrite-N, traces; and ammonium-N, 4.20 mg kg⁻¹ of soil.

Fertilizers used were urea and neem oil coated urea.

Fertilizer dose was 200 ppm of urea-N.

Coating. The calculated amount of 20% acetonetic solution of neem oil was sprayed on the urea to obtain the required dose (5000 ppm of urea-N) of the neem oil, and the coated urea was thoroughly mixed and air-dried before storage.

Treatments. The experiments were laid out following a complete randomized design with three replicates. Fifty grams of air-dried, finely ground and sieved (2 mm) soil was taken in 250 mL capacity beakers and mixed with prilled urea and/or neem oil coated urea to have 200 ppm of urea-N. Nitrapyrin (50000 ppm of urea-N) was used as a reference inhibitor. The contents of beakers were mixed and incubated at 27 °C at a moisture level of 50% of the water-holding capacity of the soil. The moisture level was maintained by adding the required amount of distilled water every alternate day throughout the study.

Sampling and Estimation of Ammonium, Nitrite, and Nitrate-N. Samples were withdrawn after 15 days of incubation and extracted with 1 M sodium sulfate solution. Ammonium, nitrite, and nitrate-N were determined by indophenol blue, modified Griess-Ilosvay (14), and phenol disulfonic acid methods (15), respectively, using a Specord 200 UV-visible spectrophotometer for the analysis.

The percent nitrification inhibition was calculated as per Sahrawat et al. (16) as follows:

$$\text{nitrification rate} = \frac{[(\text{NO}_3^- \text{-N} + \text{NO}_2^- \text{-N}) \times 100] / (\text{NH}_4^+ \text{-N} + \text{NO}_3^- \text{-N} + \text{NO}_2^- \text{-N})}{\text{NO}_3^- \text{-N} + \text{NO}_2^- \text{-N}}$$

$$\% \text{ nitrification inhibition (NI)} = \frac{[\text{nitrification rate in control} - \text{nitrification rate in sample}] \times 100}{\text{nitrification rate in control}}$$

Statistical Analysis. The data were subjected to analysis of variance (17). The treatment means were compared using CD_{5%}.

Physicochemical analysis of different neem oils was done as per the specifications laid out by the Bureau of Indian Standards (BIS) [IS: 548 (Part I)-1964].

Determination of Iodine Value. *Standardization of Wij's Iodine Monochloride Solution.* Iodine monochloride (10 mL) was dissolved in about 1800 mL of glacial acetic acid and shaken vigorously. A portion of this solution (5 mL) was mixed with potassium iodide solution (10%, 10 mL). The resulting mixture was titrated against standard sodium thiosulfate solution (0.1 N) using starch solution as an internal indicator.

Procedure. Neem oil (25 mL) warmed to 25 °C was passed through a filter containing anhydrous sodium sulfate (5 g). The moisture-free neem oil was weighed accurately in the range of 0.2538–0.3173 g into a clean dry 500 mL iodine flask to which carbon tetrachloride (25 mL) was added. The mixture was agitated to dissolve the contents.

The standard Wij's solution (25 mL) was added, and the glass stopper, after wetting with potassium iodide solution, was replaced. It was swirled thoroughly for mixing and allowed to stand for 1 h. A blank test was carried out simultaneously under similar experimental conditions. After 1 h, potassium iodide solution (10%, 15 mL) and distilled water (100 mL) were added and the stopper was also rinsed. The liberated iodine was titrated against the standard sodium thiosulfate solution (0.1 N) using starch solution as an internal indicator. The calculation of iodine value was as performed as follows:

$$\text{iodine value} = 12.69(B - S)N/W$$

B is the volume in mL of standard sodium thiosulfate solution required for the blank, *S* is the volume in mL of standard sodium thiosulfate solution required for the sample, *N* is the normality of the standard sodium thiosulfate solution, and *W* is the weight in g of the material taken for the test.

Determination of Saponification Value. *Alcoholic Potassium Hydroxide Solution.* Potassium hydroxide (35–40 g) was dissolved in 20 mL of distilled water, and aldehyde-free rectified spirit was added to make up to 1 L. The solution was allowed to stand overnight. The clear liquid was decanted off and kept in a tightly closed bottle.

Procedure. The moisture-free neem oil (1.5–2.0 g) was weighed accurately and taken in a conical flask. The alcoholic potassium hydroxide (25 mL) was added, and the resulting mixture was refluxed for up to 1 h, by which time the saponification would be complete as indicated by the absence of any oily drops and the appearance of a clear solution. After cooling of the flask and condenser, the inside of the condenser was washed with about 10 mL of hot ethanol. The aliquot was titrated against standard hydrochloric acid solution (0.1 N). The calculation was done as follows:

$$\text{saponification value} = 56.1(B - S)N/W$$

B is the volume in mL of standard hydrochloric acid required for the blank, *S* is the volume in mL of standard hydrochloric acid required for the sample, *N* is the normality of the standard hydrochloric acid, and *W* is the weight in g of the material taken for the test.

Determination of Acid Value. A suitable quantity (6–8 g) of moisture-free neem oil was weighed accurately in a 200 mL conical flask. Hot ethyl alcohol (50 mL) and about 1 mL of phenolphthalein indicator solution were added. The mixture was boiled for about 5 min and titrated while as hot as possible with standard aqueous potassium hydroxide solution (0.1 N) with vigorous shaking during titration. The acid value was calculated as follows:

$$\text{acid value} = 56.1VN/W$$

V is the volume in mL of standard potassium hydroxide solution used, *N* is the normality of the standard potassium hydroxide solution, and *W* is the weight in g of the material taken for the test.

Analysis of Meliacin Content in Neem Oils by Gravimetry. The procedure of Singh et al. (18) was used. Neem oil (15 mL), *n*-hexane (50 mL), and methanol/water (95:5, 50 mL) were transferred to a 250 mL capacity separating funnel. The contents were given a thorough shaking for 2 min and set aside. The lower layer was drained into a 250 mL capacity conical flask. The upper organic layer was twice partitioned with aqueous methanol (95:5, 50 mL × 2), and the aqueous methanolic layer was pooled, once it had been washed with *n*-hexane (25 mL) to free adhering oil. It was distilled under rotary evaporator to one-fifth of the total volume. It was cooled and transferred to a 100 mL capacity separating funnel. The aliquot was extracted with freshly distilled ethyl acetate (250 × 2), and the upper ester layer was collected over anhydrous sodium sulfate. The meliacin fraction was obtained as a dark brown viscous oil. The weight of this fraction from each oil was recorded. The experiment was repeated three times, and the mean weight is mentioned here.

RESULTS AND DISCUSSION

Effect of Neem Oils on N Dynamics. Results obtained in the *in vitro* soil incubation study are reported in Table 1.

Table 1. Effect of Different Neem Oils on Nitrification after 15 Days of Incubation

oil	ammonium-N (ppm)	nitrite-N (ppm)	nitrate-N (ppm)	nitrification inhibition (%)
Cold-Pressed Neem Oil				
A	45.7	0.41	118.0	16.3
C	40.7	0.36	106.3	16.0
L	38.3	0.44	133.3	9.8
M	32.7	0.45	142.7	5.5
N	31.7	0.45	144.3	4.7
R	48.7	0.41	136.3	14.4
S	43.7	0.39	142.3	11.1
T	40.3	0.41	145.0	9.1
W	40.3	0.44	144.3	9.2
Expeller Grade Neem Oil				
B	47.0	0.32	114.3	17.7
D	57.0	0.25	101.0	25.7
E	56.7	0.23	96.0	27.0
F	38.0	0.35	98.3	16.3
I	36.7	0.41	146.0	7.2
K	30.7	0.44	142.7	4.4
U	37.3	0.41	147.0	7.4
V	45.3	0.42	138.7	12.4
X	43.0	0.44	145.7	10.3
Y	55.3	0.32	126.3	19.2
Z	55.3	0.30	120.7	20.4
Solvent-Extracted Neem Oil				
G	42.0	0.33	94.0	19.7
O	36.3	0.40	107.3	13.2
J	31.0	0.44	146.3	4.2
Neem Oil Formulations				
P	58.7	0.20	88.0	30.3
Q	52.3	0.21	89.0	26.9
NP ^a	165.0	0.20	20.0	87.5
urea	23.7	0.39	147.0	
CD	6.5	0.01	8.4	5.4

^a NP, nitrapyrin.

Status of Ammonium-N in Treatments. *Cold-Pressed (CP) Neem Oils.* The treatments with these oils had ammonium-N contents in the range of 31.7–48.7 ppm. Oil R showed the highest (48.7 ppm) followed by oils A (45.7 ppm), S (43.7 ppm), C (40.7 ppm), T (40.3 ppm), W (40.3 ppm), L (38.3 ppm), M (32.7 ppm), and N (31.7 ppm).

Expeller Grade (EG) Neem Oils. The treatments containing these oils had ammonium-N contents in the range of 30.7–57.0 ppm. Oil D showed the highest content (57.0 ppm) followed by oils E (56.7 ppm), Y (55.3 ppm), Z (55.3 ppm), B (47.0 ppm), V (45.3 ppm), X (43.0 ppm), F (38.0 ppm), U (37.3 ppm), I (36.7 ppm), and K (30.7 ppm).

Solvent-Extracted Oils. These treatments had 31.0–42.0 ppm of ammonium-N content, and oil G (42.0 ppm) was found to be the most effective in conserving the ammonium-N followed by oils O (36.3 ppm) and J (31.0 ppm).

Neem Oil Formulations. Of two formulations tested, P showed higher ammonium-N content (58.7 ppm) than Q (52.3 ppm). Both formulations were found to be statistically at par.

Overall Comparison. All of the neem oils showed higher ammonium-N contents (30.7–58.7 ppm) after 15 days of sampling than urea control (23.7 ppm). Nitrapyrin had the highest ammonium-N content (165 ppm). Among the neem oils, neem oil formulation P performed the best (58.7 ppm) followed by expeller grade neem oils D (57.0 ppm), E (56.7 ppm), Y (55.3 ppm), and Z (55.3 ppm) and neem oil formulation Q (52.3 ppm), and these treatments were statistically at par. The next in order were oils R (48.7 ppm), B (47.0), A (45.7 ppm), V (45.3 ppm), and X (43.0 ppm). These treatments were also

statistically at par with each other as evident from CD value. The performance of oils G, C, T, W, L, F, U, O, M, J, and K was superior to that of urea alone, showing ammonium-N contents in the range of 30.7–42.0 ppm.

Status of Nitrite-N in Treatments. Nitrite-N content was insignificant (<0.5 ppm) throughout the experiment in all of the treatments. It is thus concluded that neem oil did not cause accumulation of nitrite-N.

Status of Nitrate-N in Treatments. *CP Neem Oils.* Treatments with these oils had nitrate-N contents in the range of 106.3–145.0 ppm. Oil C showed the lowest nitrate-N content (106.3 ppm) followed by oils A (118.0 ppm), L (133.3 ppm), R (136.3 ppm), S (142.3 ppm), M (142.7 ppm), N (144.3 ppm), W (144.3 ppm), and T (145.0 ppm).

EG Neem Oils. The treatments containing these oils had nitrate-N contents in the range of 96.0–147.0 ppm. Oil E showed the lowest nitrate-N content (96.0 ppm) followed by oils F (98.3 ppm), D (101.0 ppm), B (114.3 ppm), Z (120.7 ppm), Y (126.3 ppm), V (138.7 ppm), K (142.7 ppm), X (145.7 ppm), I (146.0 ppm), and U (147.0 ppm).

Solvent-Extracted Oils. These treatments had 94.0–146.3 ppm of nitrate-N contents, and oil G (94.0 ppm) was found to be the most effective in decreasing the nitrate-N content followed by oils O (107.3 ppm) and J (146.3 ppm).

Neem Oil Formulations. Of two formulations tested, P showed a little lower nitrate-N content (88.0 ppm) than Q (89.0 ppm), but both were statistically at par with each other.

Overall Comparison. All treatments caused varied accumulation of nitrate-N (88.0–147.0 ppm). Nitrapyrin caused the lowest nitrate-N production (20.0 ppm). Among the neem oils, neem formulations P and Q were found to be most effective, causing 88.0–89.0 ppm of nitrate N, and the next in order of efficacy were oils G (94.0 ppm) and E (96.0 ppm). The other effective neem oils were F (98.3 ppm), D (101 ppm), C (106.3 ppm), and O (107.3 ppm), causing 27.0–33.1% reductions in nitrate-N formation as compared to urea control (147 ppm). Oils B, A, Z, Y, L, and R caused only marginal decreases in nitrate-N, but were superior to urea alone. The remaining oils were statistically at par with urea alone.

Nitrification Inhibition (NI) by Treatments. *CP Neem Oils.* All of the CP grade neem oils caused NI in the range of 4.7–16.3%. Oil A was the best with NI of 16.3% and statistically at par with oils C (16.0%), R (14.4%), and S (11.1%). The next statistically similar group includes oils L (9.8%), W (9.2%), T (9.1%), M (5.5%), and N (4.7%).

EG Neem Oils. All of the EG grade neem oils caused NI in the range of 4.4–27.0%. Oil E caused the highest NI (27.0%) followed by oils D (25.7%), Z (20.4%), Y (19.2%), B (17.7%), F (16.3%), V (12.4%), X (10.3%), U (7.4%), I (7.2%), and K (4.4%).

Solvent-Extracted Oils. These caused 4.2–19.7% NI. Oil G (19.7%) was found to be the most effective followed by oils O (13.2%) and J (4.2%).

Neem Oil Formulations. Of the two formulations tested, P showed a higher NI (30.3%) than Q (26.9%), but they were statistically at par.

Overall Comparison. Nitrapyrin showed maximum nitrification inhibition (87.5%), but its dose was 10 times higher than that of other treatments. Among the neem oils, neem oil commercial formulations (P and Q) and EG oils (D and E) were found to be the best and statistically equivalent, causing 30.9, 26.9, 25.7, and 27.0% nitrification inhibition, respectively. The next in order of efficacy were oils Z (20.4%) ≈ G (19.7%) ≈ Y (19.2%) ≈ B (17.7%) ≈ A (16.3%) ≈ F (16.3%) ≈ C (16.0%)

Table 2. Meliacin Content of Neem Oils

oil	meliacin content (%)	oil	meliacin content (%)	oil	meliacin content (%)
A	10.00	H	19.40	M	7.8
B	4.70	I	5.4	N	3.50
C	9.19	J	9.80	O	15.32
D	11.00	K	16.42	P	16.60
E	7.80	L	4.17	Q	11.4

Table 3. Physicochemical Characterization of Different Neem Oils^a

oil	iodine value	saponification value	acid value
A	75.12	299.56	13.15
B	51.90	192.42	17.68
C	61.23	206.54	20.92
D	56.76	189.11	12.25
E	26.92	234.86	30.99
F	69.05	228.06	16.70
G	37.16	218.00	12.87
I	56.78	346.38	28.44
J	60.02	241.97	21.23
K	67.20	252.05	28.02
L	61.62	238.49	05.12
M	64.58	199.54	06.45
N	52.63	185.27	09.74
O	52.73	223.53	41.14
P	64.27	196.19	21.75
Q	41.44	196.59	07.99
R	56.41	315.38	3.53
S	57.25	294.59	39.02
T	52.53	226.22	82.16
U	59.45	144.49	8.41
V	53.95	131.11	16.70
W	61.78	150.52	32.73
X	81.99	122.99	7.34
Y	60.72	49.96	3.63
Z	74.07	64.41	39.08

^a CP neem oil from different sources (A, C, L, M, N, R, S, T, W); EG neem oil from different sources (B, D, E, F, I, U, V, X, Y, Z); solvent-extracted neem oils from different sources (G, J, O); neem formulations (P, Q).

≈ R (14.4%). The other neem oils that showed ≥10% nitrification inhibition were O (13.2%), V (12.4%), S (11.1%), and X (10.3%). The rest of the oils showed 4.0–9.8% nitrification inhibition.

Correlation Analysis of NI Activity versus Physicochemical Properties of Neem Oils. The meliacin content of neem oils varied from 5.4 to 16.6% (Table 2). Correlation of meliacin content versus NI activity of neem oil gave $r = 0.27$, implying that there is a direct influence of meliacin content on NI activity. Physicochemical analysis of different neem oils was done as per the specifications prescribed by the Bureau of Indian Standards [IS: 548 (Part I)-1964]. The oils showed a lot of variation in their physicochemical behavior as seen in Table 3. A correlation matrix of these values vis-à-vis NI is given in Table 4. It is seen that NI was found to be negatively correlated with saponification and iodine values. This implies that for this target activity, oils rich in meliacins and saturated triglycerides would be preferable. As a sequel to this study, we have planned to evaluate meliacins for their effect on nitrification. It is also desired to identify a suitable meliacin as a marker for ensuring the authenticity and quality of neem oil.

Compliance of BIS Specifications. A perusal of data in Table 3 with reference to specifications set by the BIS reveals that the iodine values of only oils A, F, K, M, P, X, and Z complied with the BIS standards (65–85). As per BIS standards only six oils (B, D, M, N, P, and Q) showed saponification values within the required limit (175–200). Fifteen oils (A–

Table 4. Correlation Coefficient Matrices between Various Physicochemical Properties and Nitrification Inhibitory Activity of Neem Oils ($n = 13$)

	nitrification inhibition	iodine value	saponification value	acid value
nitrification inhibition	1.000			
iodine value	-0.408	1.000		
saponification value	-0.326	0.242	1.000	
acid value	-0.041	-0.203	0.374	1.000

Table 5. Scorecard of Active Oils with Respect to Compliance to BIS Specifications

oil	iodine value	acid value	saponification value
P	+	+	-
Q	-	+	+
D	-	+	+
E	-	-	-
G	-	-	+
Y	-	-	+
Z	+	-	-
score	2/7	3/7	4/7

D, F, G, L, M, N, Q, S, U, V, X, and Y) possessed acid values within the limits as per the BIS (<20). We verified whether the BIS specifications are adequate for neem oils as raw materials in the production of NOCU. Table 5 lists the score card on BIS compliance. Of the seven most active oils, only 2, 3, and 4, respectively, complied with respect to iodine value, acid value, and saponification value. Iodine value appears to be a poor test for these oils. Considering the poor influence of acid value on NI, we must therefore rely more on saponification values. Furthermore, we find that meliacin content is also critical for NI as discussed above. The present BIS specifications do not include this factor. Therefore, there is a need to introduce new BIS specifications incorporating these parameters for neem oils as raw materials of NOCUs.

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