3. MATERIALS AND METHODS
3.1. Soil

Sandy loam soil, from agricultural fields of Samathagram, a village near the University campus which represents the semi-arid region of Andhra Pradesh, was collected to a depth of 12 cm. Soil was sieved through a 2-mm mesh before use. The measured physico-chemical characteristics of the soil are presented in Table 2.

3.2. Neem cake

The locally available commercial deoiled neem cake, the kernel residue after oil extraction of seeds of neem plant (*Azadirachta indica* L.) was used in this experiment. The following is the chemical composition of the neem cake (Patnalk, 1961).

- Nitrogen : 5.2 - 5.6%
- $P_2O_5$ : 1.1%
- $K_2O$ : 1.5%

3.3. Mulberry saplings

In the present experiment, K2 (Kanva-2), a variety of mulberry (*Morus alba* L.) was used. It is an open-pollinated hybrid (OPH). It grows vigorously and responds well to agronomic practices and performs well both under rainfed and irrigated conditions (Mallikarjunappa et al., 1992). The saplings were collected from the
### Table 2. Physico-chemical characteristics of the soil used in the present study

<table>
<thead>
<tr>
<th>Soil characteristic</th>
<th>Mean value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sand (%)</td>
<td>61.5</td>
</tr>
<tr>
<td>Silt (%)</td>
<td>32.2</td>
</tr>
<tr>
<td>Clay (%)</td>
<td>6.3</td>
</tr>
<tr>
<td>$pH^a$</td>
<td>7.7</td>
</tr>
<tr>
<td>Organic matter$^b$</td>
<td>0.912</td>
</tr>
<tr>
<td>Total N (%)$^c$</td>
<td>0.056</td>
</tr>
<tr>
<td>$NH_4^+ - N$ (µg g$^{-1}$ soil)$^d$</td>
<td>9.250</td>
</tr>
<tr>
<td>$NO_2^- - N$ (µg g$^{-1}$ soil)$^e$</td>
<td>0.540</td>
</tr>
<tr>
<td>$NO_3^- - N$ (µg g$^{-1}$ soil)$^f$</td>
<td>1.182</td>
</tr>
<tr>
<td>Electrical conductivity</td>
<td>$120 \times 10^{-6}$ µmhos</td>
</tr>
</tbody>
</table>

$^a$ 1:1.25 soil : water slurry

$^b$ Walkley - Black method (Jackson, 1971).

$^c$ Micro-Kjeldahl method (Jackson, 1971).

$^d$ Nesslerisation method (Jackson, 1971).

$^e$ Diazotisation method (Barnes and Folkard, 1951).

$^f$ Brucine method (Ranney and Bartlett, 1972).
Regional Sericulture Research Station, Anantapur, Andhra Pradesh.

3.4. Pot culture experiment

Soil samples (4 kg) were thoroughly mixed with powdered neem cake at a rate of 100 kg/acre, and placed in earthen pots (10" x 12"). Two saplings of a two-month-old Kanva-2 mulberry variety were planted in each pot. The saplings were pruned to a height of 6 cm. The potted plants without neem cake-amendment served as untreated controls. All the plants were watered regularly for every 5 to 6 days.

To a set of neem cake amended pots, an application of the neem cake was given 90 days after first application. This is in view of the recommendations made for an effective control of root-knot nematodes in mulberry cultivation (Sikdar et al., 1986). At the time of second application, the required quantity of neem cake was made into a slurry with water and was added to the potting medium 2 cm below the top soil. At least five replicate pots were maintained for each treatment.

Mulberry plants were grown under glass house conditions (31 ± 9°C day/26 ± 2°C night) in a randomized block design up to 90 days after the second application of neem cake. Triplicates of each treatment were withdrawn for sampling the soil 45 and 90 days after the
first and second application of neem cake.

3.4.1. Morphological data

Before the soil was withdrawn from the pots for sampling, certain plant growth parameters such as plant height, leaf number were recorded besides determining the leaf area. In order to determine the leaf area of mulberry, the fully expanded second or third leaf from the apex was collected from the plants of both treatments. The outline of the leaves were plotted on a centimeter graph sheet and the leaf area was calculated. The leaf area was expressed in square centimeters.

3.4.2. Soil sampling

At the appropriate time of sampling (45 and 90 days after first and second application of neem cake), the potted plants were gently uprooted and the entire soil (both rhizosphere and non-rhizosphere) was collected and thoroughly mixed. The soil samples thus collected from triplicates of unamended and neem cake-amended pots, were kept in polyethylene bags and stored at 0°C in a deep-freezer before being utilized for the determination of microbial populations, elemental transformations and enzyme activities.
3.5. Effect of neem cake on microbial populations in soil

A comparison was made on the microbial populations such as total bacteria, nitrifiers, the free-living diazotroph *Azospirillum* sp., and fungi in samples collected from unamended and neem cake-amended soil.

3.5.1. Total bacteria

One gram soil samples for each treatment was used to prepare 10-fold soil suspension following the method of serial dilution (Waksman, 1922; Allen, 1959). Aliquots (0.1 ml) from $10^{-6}$ to $10^{-9}$ soil dilutions were used to spread on to nutrient agar medium with the following composition.

- **Beef extract**: 3.0 g
- **Peptone**: 5.0 g
- **Agar**: 15.0 g
- **Distilled water**: 1000 ml

Triplicate petriplates, maintained for each soil dilution, were incubated at 37°C in a bacteriological incubator for 48 hours and the number of bacterial colonies developed on the medium were recorded. The population estimate of soil bacteria was expressed per gram oven-dried soil.
3.5.2. Nitrifiers

The influence of neem cake on population of nitrifiers was determined as follows. Triplicate soil samples were used for estimating the population density of nitrifiers employing the two-fold serial dilution by the most-probable number (MPN) method (Muralikrishna and Venkateswarlu, 1984).

Ten gram soil samples of each treatment were suspended in 100 ml sterilized distilled water separately in 250 ml Erlenmeyer flasks and thoroughly agitated in a wrist-action shaker for 30 minutes. Two-fold serial dilutions, up to 1:1,28,000 (soil to water ratio) were prepared further from this soil suspension. Five MPN tubes, each containing 5 ml steam-sterilized mineral salts medium with the following composition (Alexander, 1965a), received 0.5 ml aliquots of the soil suspensions from dilutions ranging from 1:1000 to 1:1,28,000.

\[
\begin{align*}
\text{NH}_4\text{SO}_4 & : 1.0 \text{ g} \\
\text{K}_2\text{HPO}_4 & : 1.0 \text{ g} \\
\text{MgSO}_4 & : 0.5 \text{ g} \\
\text{NaCl} & : 2.0 \text{ g} \\
\text{FeSO}_4 & : \text{Traces} \\
\text{CaCO}_3 & : 1.0 \text{ g} \\
\text{Distilled water} & : 1000 \text{ ml}
\end{align*}
\]
After 72 hours incubation at 37°C in a bacteriological incubator, the MPN tubes were withdrawn for determining the viable count estimates of nitrifiers (both nitrosifying and nitrifying) by testing for the presence of nitrite following the method described elsewhere.

The MPN tubes which exhibited negative response to the test were treated with zinc dust and scored positive if developed pink colour. MPN values were calculated by referring to the tubes of Fisher and Yates (1963).

3.5.3. *Azospirillum* Sp.

The populations of *Azospirillum* sp. in mulberry-grown soil samples were also estimated by the MPN technique following ten-fold serial dilution and the numbers were calculated using the probability tables (Alexander, 1965b). Five milliliter portions of sterile N-free semi-solid malate medium with the composition given below, selective for *Azospirillum lipoferum* (Dobereiner et al., 1976), taken in five MPN tubes, were inoculated with 0.5 ml aliquots of the suspensions from $10^{-3}$ to $10^{-7}$ soil dilutions and incubated at 37°C.

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Malic acid</td>
<td>5.0 g</td>
</tr>
<tr>
<td>KOH</td>
<td>4.0 g</td>
</tr>
<tr>
<td>$K_2HPO_4$</td>
<td>0.5 g</td>
</tr>
<tr>
<td>$MgSO_4$</td>
<td>0.2 g</td>
</tr>
</tbody>
</table>
MPN tubes in which a typical white pellicle developed a few millimeters below the surface of the medium within 36 hours were scored positive for Azospirillum sp. Microscopic examination of the cultures revealed the characteristics rods with flat droplets and very active spiral movements.

3.5.4. Fungi

Soil populations of fungi were estimated by the serial dilution plating technique (Waksman, 1922) following 10-fold dilution. Aliquots (0.1 ml) of the soil suspension from the dilutions of $10^{-4}$ to $10^{-6}$ were spread on to solidified Czapex-Dox agar medium containing Rose-bengal (Thom and Raper, 1945; Martin, 1950), the composition of which is as follows:
Triplicate petriplates were maintained for each soil dilution. After incubating the plates at 37°C in a bacteriological incubator for 72 hours, the number of fungal colonies developed on the medium were recorded. The population estimate of soil fungi was expressed per gram of oven-dried soil.

3.6. Effect of neem cake on ammonification and nitrification in soil

3.6.1. Ammonification

To determine the rate of ammonification, 10-g portions of the unamended and neem cake-amended soil samples were supplemented with 1000 ppm nitrogen as analytical grade peptone. The soil samples were then homogenised to distribute the source of nitrogen and enough distilled water was added to maintain at 60% water-holding
capacity (WHC). Moisture levels were restored to their initial values during incubation period. Triplicate samples of each treatment were withdrawn after 10 and 20 days to determine the rate of ammonification, measured in terms of the quantities of NH$_4^+$ and also NO$_2^-$ and NO$_3^-$ as followed by Tu (1973).

3.6.2. Nitrification

The procedure adopted for the determination of nitrification was same as with the ammonification. Portions (10 g) of samples from unamended and neem cake-amended soils were taken separately in test tubes (25 x 200-mm) and supplemented with 200 ppm N in the form of AnalaR grade ammonium sulphate. After 10, 20 days of incubation at room temperature (28 ± 4°C), triplicate samples were withdrawn for estimating nitrite and nitrate as described elsewhere.

3.6.3. Extraction of the soil samples for estimating ammonium, nitrite and nitrate.

Triplicates of either peptone-amended or ammonium sulphate-amended soil samples were withdrawn at desired intervals and extracted with 100 ml 2 M KCl by thoroughly agitation in a wrist-action shaker. After 30 minutes of shaking, the soil suspensions were filtered through Whatman No.1 filter paper and appropriate aliquots
of the filtrate were used for the estimation of ammonium, nitrite and nitrate.

a. Estimation of ammonium

The ammonium (NH$_4^+$-N) formed in peptone amended soil samples was analysed by nesslerisation (Jackson, 1971). To suitable aliquots of the soil extract, 0.5 ml of the Nessler's reagent was added and the volume was made up to 7 ml. The yellow colour developed was read at 495 nm in a Spectronic-20 spectrophotometer (Baush and Lomb). The amount of ammonium was calculated by referring to a calibration curve prepared with the standard solution of known ammonium concentrations.

b. Estimation of nitrite

Nitrite was estimated by diazotisation following the method of Barnes and Folkard (1951). Suitable aliquots from the filtrate of the soil extract were pipetted into test tubes, and 1 ml of 1% sulphanilamide in 1N HCl was added and shaken thoroughly. To the coloured diazo compound so formed was added 1 ml of 0.12% N-(1-naphthyl)ethylenediamine dihydrochloride in distilled water. The colour was allowed to develop for 25 minutes, after which the volume was made up to 25 ml with distilled water. The absorbance of the pink coloured solution was read at 520 nm in a Spectronic-20
spectrophotometer. The amount of nitrite was calculated by referring to a calibration curve prepared with the standard solution of known nitrite concentrations.

c. Estimation of nitrate

The nitrate ($\text{NO}_3^-$-N) produced due to the activity of soil microorganisms was determined by the method of Ranney and Bartlett (1972). Three drops of brucine reagent (2 g brucine dissolved in 50 ml methanol) were added to suitable aliquots of the soil extract followed by 2 ml of concentrated sulphuric acid. The solution was mixed by vortexing and placed in the dark for 30 minutes to ensure full colour development, after which the volume was made up to 10 ml with distilled water, and the yellow colour was read at 410 nm in a Spectronic-20 spectrophotometer.

3.7. Effect of neem cake on enzyme activities in soil

3.7.1. Amylase and invertase

In triplicate samples of both unamended and neem cake-amended soil, amylase and invertase were assayed, as detailed earlier by Tu (1982) and Chendrayan et al. (1980), respectively. The method employed for the assay of amylase and invertase was essentially the
same developed by Cole (1977). The soil samples were transferred to 250 ml Erlenmeyer flasks and 2 ml toluene was added. After 15 minutes, 12.5 ml of 0.2 M acetate phosphate buffer (pH 5.5) containing either 2% starch (for amylase) or 18 mM sucrose (for invertase) were added to the soil samples and the flasks were closed with cotton plugs and held for 24 hours and 72 hours at 30°C (for amylase activity), or for 24 hours and 48 hours at 30°C (for invertase activity). Soil extracts were passed through Whatman No.1 filter paper and glucose in the filtrate was assayed (Nelson, 1944), at 510 nm in a Spectronic-20 spectrophotometer.

3.7.2. Protease

In triplicate samples of the unamended and neem cake-amended soil, protease was assayed (Spier and Ross, 1975) at intervals of 45 and 90 days. Unamended and neem cake-amended soil samples (2 g) were incubated for 2 hours at 30°C with 10 ml of 0.1 M tris-(2-amino-2-(hydroxymethyl)propane-1:3-diol, pH 7.5) containing sodium caseinate (2% w/v). Four milliliters of aqueous solution (17.5% w/v) of trichloro acetic acid was then added and the mixture was centrifuged. A suitable aliquot of the supernatant was treated with 3 ml of 1.4 M NaCO₃ followed by the addition of 1.0 ml Folin-Cicalteu reagent
(33.3% v/v). The blue colour was read after 30 minutes at 700 nm in a spectrophotometer. Tyrosine was used as a standard.

3.7.3. Phosphatase

Triplicate samples of both unamended and neem cake-amended soil were used for the assay of phosphatases (Tabatabai and Bremner, 1969). Each soil sample was treated with 6 ml of 0.1 M maleate buffer (pH 6.5) and 2 ml of 0.03 M p-nitrophenol phosphate. After incubation for 30 minutes at 37°C, the tubes were placed on ice before the soil extracts were filtered through Whatman No.1 filter paper. To suitable aliquots of the extract, 1 ml of 5 M CaCl$_2$ and 4 ml of 0.5 M NaOH were added, and the yellow colour developed was read at 405 nm.

3.7.4. Urease

Triplicate samples of both unamended and neem cake-amended were assayed for urease following the phenol - hypochlorite method (Fawcett and Scott, 1960). At desired intervals, one ml of 3% urea and 2 ml of 0.1 M phosphate buffer (pH 7.1) were added to one-gram soil. After incubation for 30 minutes at 37°C in a water bath shaker, the tubes were placed in the ice until the ammonia extracted with 10 ml of 2 M KCl. Five milliliters of phenol - sodium nitroprusside solution and 3 ml of 0.02 M
sodium hypochlorite were added to 4 ml of the filtrate. The mixture was shaken, incubated for 30 minutes in the dark, and the developed yellow colour was measured at 630 nm.

3.8. Statistical analysis

In all cases, the results of neem cake amendments were contrasted with unamended controls and the significant differences ($P \leq 0.05$) between values of samplings either for single application or two applications were performed using Duncan's new multiple range (DMR) test (Duncans, 1955).