MATERIALS AND METHODS
Vigna unguiculata L. variety Russian giant was taken as experimental plant. The seeds were procured from the Andhra Pradesh Agricultural Department, Anantapur-515 003, India. The seeds were sown in pots. The experiments were conducted in leaves of 15 days old plants. The leaves were detached from the plant, washed in deionized water, and were surface sterilized with 0.1% mercuric chloride solution for 30 seconds. They were then washed with deionized water. The leaves were placed in petri dishes of 20 cm diameter containing distilled water or metal solutions. Ten leaves were placed in each petri dish.

The heavy metals studied were CdCl₂ and NiCl₂. Different concentrations namely 1 µM, 10 µM, 100 µM and 1 mM solutions of the above salts were prepared in distilled water and distilled water alone served as control. Three petri dishes were placed for each concentration of metal.

One set of petri dishes was kept under a light intensity of approximately 150 Wm⁻² and at a temperature of 27°C ± 3, and another set of petri dishes was kept under darkness. The solutions were replaced every day with fresh ones. The samples were taken after 24 h, 48 h, 72 h and 96 h of incubation. Three experiments were conducted at different times.
Extraction and estimation of chlorophyll pigments:

Total chlorophyll content was estimated according to the method of Arnon (1949). Leaf material was taken from petri dishes, washed and blotted to dry. 200 mg of leaf material was homogenized in a prechilled mortar using 80% cold acetone. The homogenate was centrifuged at 3000 rpm in a Remi centrifuge and the supernatant was collected. The sediment was reextracted with 80% acetone until all the chlorophyll was removed from the sediment. The acetone supernatants were pooled and made up to a known volume with 80% cold acetone. The absorbance of the acetone extract was measured at 645 nm and 663 nm in ELICO model CL 24 Junior Spectrophotometer using 80% acetone as blank. Total chlorophyll, chlorophyll \( a \) and \( b \) contents were calculated using the following formulae:

Total chlorophylls =

\[ 20.2 \times O.D \text{ at } 645 \text{ nm} + 8.02 \times O.D. \text{ at } 663 \text{ nm} \]

Chlorophyll \( a \) =

\[ 12.7 \times O.D. \text{ at } 663 \text{ nm} - 2.69 \times O.D. \text{ at } 645 \text{ nm}. \]

Chlorophyll \( b \) =

\[ 22.9 \times O.D. \text{ at } 645 \text{ nm} - 4.68 \times O.D. \text{ at } 663 \text{ nm}. \]
Estimation of proteins:

The total protein content was estimated in control and treated leaves of *Vigna unguiculata* by Biuret method according to Layne (1957).

Stomatal frequency:

Epidermal strips of about 1.0 x 0.5 mm were taken from control and metal treated leaves and were mounted on a microscopic slide in the respective metal solution. Stomatal frequency was calculated by counting the number of stomata under a microscope (10 X x 40 X) in the microscopic field area. Similarly, the number of totally closed, partially closed and fully opened stomata were counted in the microscopic field and percentage was calculated (Lloyd, 1921).

\[
\text{Stomatal frequency} = \frac{\text{Number of stomata}}{\text{Area of microscope field}}
\]

Area of the microscope field:

The radius of the microscopic field was found under the high power (10 X x 40 X) of the microscope using the stage micrometer. By adopting the formula \( \pi r^2 \), the area of microscopic field was calculated and expressed in square millimeters (mm²).

Extraction of proteins:

200 mg of leaf tissue was extracted into 80% hot ethanol by macerating in a mortar with pestle. The
homogenate was transferred into centrifuge tubes and centrifuged at 2000 g for 20 minutes and the supernatant was discarded. The pellet was suspended in a suitable volume of 10% trichloro acetic acid (TCA). Again it was centrifuged and the supernatant was discarded. This process was repeated twice. The pellet was then reextracted with absolute ethanol and with hot ethanol - ether mixture (3:1). Every time the supernatant was discarded after centrifugation. Protein was estimated from the pellet.

Determination of protein:

The pellet was suspended in 2.0 ml of 1 N NaOH in test tubes at 100°C for 4.5 minutes and 3.0 ml of Biuret reagent was added to it. The contents were mixed and incubated for 10 minutes in a water bath set at 37°C. The test tubes were cooled and the absorbance was measured at 540 nm against reagent blank in Junior Spectrophotometer (ELICO model CL 24). Protein content was calculated from a standard curve prepared from bovine serum albumin (BSA).

Reagents:

10% TCA:

10 g of trichloroacetic acid was dissolved in distilled water and made upto 100 ml with distilled water.
Ethanol-ether mixture (3:1):

75 ml of ethanol and 25 ml of ether were mixed.

1 N NaOH:

4 g of NaOH was dissolved in distilled water and made up to 100 ml.

Biuret reagent:

3 g of CuSO$_4$$\cdot$5H$_2$O and 9 g of sodium potassium tartrate were dissolved in 500 ml of 0.2 N NaOH solution and 5 g of potassium iodide was added to it and made up to 1 litre with 0.2 N NaOH solution.

Nucleic acids:

The extraction of nucleic acids was done according to Osborne (1962).

The tissue was extracted with hot 80% ethanol. The tissue was cut into pieces and was plunged into boiling ethyl alcohol and was allowed to boil for 5-10 min. The tissue was then macerated in a mortar with pestle. The homogenate was transferred into centrifuge tubes. It was centrifuged at 2000 rpm for 20 min and the supernatant was discarded. The pellet was suspended into a suitable volume of 5% TCA at 0-2°C for 15 min. After incubation, it was centrifuged and the supernatant was discarded. This process
was repeated twice. The pellet was reextracted with absolute ethanol and twice with hot ethanol-ether mixture, every time discarding the supernatant after centrifugation.

The pellet was suspended in a suitable volume of 0.3 N KOH and incubated for 16 h at 37° C. Then it was centrifuged at 2000 rpm for 15 min and the supernatant was collected. The residue was washed twice with distilled water and the supernatants were collected and made up to known volume.

Separation of DNA and RNA:

The nucleic acid extract was acidified to pH 2.0 with perchloric acid. This precipitated the deoxyribonucleic acid and excess of KClO₄. This was centrifuged at 2000 rpm for 15 min and the supernatant and residue were collected, which contained RNA and DNA respectively.

Estimation of DNA:

The residue containing DNA was suspended in suitable volume of 5% TCA at 0° C and centrifuged at 2000 rpm for 15 min and the supernatant was discarded. The above process was repeated twice. The residue was washed with absolute ethanol and then with ethanol-ether mixture. The residue was suspended in 0.5 N HClO₄ and incubated at 90° C for 7 min in a constant temperature water bath. It was
centrifuged and the supernatant was collected. The residue was washed twice with water and the supernatant was pooled up and made up to known volume. To the above, an equal volume of 1 N KOH was added to precipitate the excess perchlorate as KClO₄. This was centrifuged at 2000 rpm for 15 min and the supernatant was collected containing DNA.

The colour reaction between deoxyribooses and diphenylamine was used for the determination of DNA (Burton, 1968).

The extract was diluted with 0.5 N HClO₄ so that the sample for analysis contains 0.02 to 0.25 umoles of DNA-phosphate per ml. 2.0 ml of the sample was pipetted out into a test tube and to that 4.0 ml of diphenylamine reagent was added. Appropriate standards and blank tubes containing the same amount of perchloric acid were maintained. The tubes were incubated at 25°C for 15 min, and then the absorbance was measured at 600 nm in a Bausch and Lomb Spectronic 88 spectrophotometer. The amount of RNA was calculated using a standard curve prepared with calf thymus DNA.

Reagents:

Aqueous acetaldehyde 1.6%:

1.0 ml of cold acetaldehyde was mixed in 50 ml of
distilled water using a prechilled pipette and was stored in a stoppered bottle. It was stable for several months at 4° C.

Diphenylamine reagent:

1.5 g of diphenylamine was dissolved in 100 ml of glacial acetic acid (stored in dark) and 1.5 ml of conc. H₂SO₄ was added to it. Just before use 0.1 ml of 1.6% aqueous acetaldehyde was added to 20 ml of the above reagent. The reagent is stable without acetaldehyde for 3 months at 4° C.

Standard solution:

Calf thymus DNA (0.3 mg/ml) was dissolved in 5 mM NaOH. The stock solution is stable for at least 6 months at 4° C. The standards were prepared by mixing equal volumes of the DNA solution with 1 N HClO₄ and heating it for 15 min at 70° C. The solution is stable for 3 weeks.

Estimation of RNA:

The supernatant obtained after the addition of HClO₄ was taken for the estimation of RNA. The supernatant was diluted to a known volume. To 1.0 ml of this supernatant, 0.3 ml of 0.2% orcinol and 3.0 ml of 0.1% ferric chloride solution were added. Then it was incubated in a boiling water bath for 1 h. After incubation the tubes
cooled and the colour intensity was read at 665 nm in a Bausch and Lomb Spectronic 88 spectrophotometer. The standards were prepared with yeast RNA and the amount of RNA was calculated from the standard curve.

Ribonuclease (E.C. 3.1.4.22):

The enzyme was extracted and assayed according to the procedure described by Naito et al. (1979).

Extraction:

200 mg of plant material was sliced and homogenized with ice cold 0.1 M phosphate buffer (pH 6.5). The homogenate was centrifuged at 2000 rpm in a refrigerated high speed centrifuge for 30 min at 0-2°C. The supernatant was assayed for RNase activity.

For determination of RNase activity, 0.2 ml of enzyme preparation was incubated with 1.0 ml of 1.5 mg/ml yeast RNA in 0.05 M phosphate buffer (pH 6.5) and 0.8 ml of the same buffer for 2 h at 37°C. At the end of the incubation, the undigested nucleic acid was precipitated by centrifugation at 2000 rpm for 15 min after the addition of 0.5 ml of chilled 25% perchloric acid containing 0.75% uranyl acetate. The supernatant was diluted to known volume before reading the absorbance at 260 nm against boiled enzyme.