MATERIALS AND METHODS

The experiments were conducted by using sulphur dioxide (SO₂) and ozone (O₃) as gaseous air pollutants for exposure of the experimental plants and fly ash as a particulate air pollutant for incorporation in soil. The effects were determined on plant growth and yield of black gram (Vigna mungo (L.) Hepper), an important leguminous crop in India and on their root nodulation by root nodule bacterium, Rhizobium sp. nodulating on black gram (Vigna mungo L.) and root colonization by VAM fungus, Glomus caledonicum (Nicol. and Gerd). Trappe and Gerdemann. Preliminary survey of fields grown with black gram in and around Aligarh showed the Glomus caledonicum was common VAM fungus infecting the roots of the plants. Isolation of spores from soil samples collected from the same field yielded higher number of Glomus caledonicum spores than other VAM fungi. Therefore, this VAM fungus was selected for the experimental work in this study. The details of materials used and methods employed in the study are given below.

VAM FUNGUS

Starter culture of VAM fungus (inoculum production)

(a) Collection of soil samples in the study

Glomus caledonicum was used as VAM fungus. In order to collect spores of G. caledonicum, fifty soil samples were collected from the crop fields in Aligarh and adjoining areas grown with black gram (Vigna mungo). Samples were collected with the help of soil auger upto depth of 15 cm underneath the plants.

(b) Isolation of spores of VAM fungi from the soil samples

Spores of VAM fungi present in the soil samples were isolated by wet sieving and decanting method (Gerdemann and Nicolson, 1963). A sample of 100 g dry soil was mixed in water (1000 ml) and the heavier particles were allowed to settle for few seconds. The liquid was poured
liquid passed through coarse sieve was collected and again passed through a series of sieves of varied size i.e. 80, 150, 250 and 300 mesh. Spores obtained on sieves were collected with water in separate beakers. Repeated washings with Ringer’s solution (NaCl 6g l⁻¹, KCl 0.1g l⁻¹ and CaCl₂ 0.1 g l⁻¹ in D.W., pH 7.4) isolated and recovered most of spores from soil detritus. Spores of VAM fungi were identified under a dissecting microscope (15X) with the help of the synoptic keys (Trappe, 1982) and the spores of G. caledonicum were separated by picking and used for pot culture. Spores were separated with a microspatula and picked up by a pasteur pipette fitted with a rubber bulb. These were surface sterilized for 2 minutes in a solution containing chloroamine T 20g/l, streptomycin 300 mg/l and tween 80 a trace amount/l in distilled water.

(c) Culture

Culture of G. caledonicum was raised on black gram (Vigna mungo) plants grown in pots under glasshouse conditions. Seeds of black gram cv. Type 9 Pantnagar were surface sterilized with 0.1% solution of HgCl₂. The surface sterilized seeds were dressed with soil-based culture of Rhizobium sp. and planted (5 seeds per pot) in 50 clay pots of 9 cm diam. containing sterilized field soil (66% sand, 24% silt, 8% clay, OM 2%, pH 7.5). Fifty spores of G. caledonicum per pot were layered at 6 and 2 cm. in each clay pot. After emergence, seedlings were thinned and one seedling was maintained in each pot. After 125 days, the plants were uprooted and the spores were isolated by wet sieving and decanting method from the pot soil. Roots were examined for the VAM colonization.

Inoculation of VAM fungus

Spores of G. caledonicum were used as inoculum for the experiments. The spores were obtained from the pot culture of G. caledonicum raised on black gram, as described above. Spores were applied at the rate of 1000 spores/pot as thin layers 6 and 2 cm below the surface of the soil before sowing the seeds in experimental pots.
ROOT NODULE BACTERIUM

Isolation of root nodule bacterium

Rhizobium sp. was isolated from root nodules present on roots of black gram plants collected from fields. After washing the root system of the plants in running water, a well-formed healthy pinkish nodule on the tap root was carefully cut out with a portion of root attached to the nodule. The nodule was surface sterilized for 5 min. in 0.1% mercuric chloride and repeatedly washed with sterilized distilled water to remove the chemical. The nodule was then washed in 70% ethyl alcohol for 3 min. followed by more washing with sterilized distilled water (Ash and Allen, 1948). The nodule was crushed with sterilized glass rod in a small aliquot of sterilized water and diluted for obtaining clear and distinct colonies. Congo red yeast-extract mannitol agar medium (CRYMA) was used for the isolations. The constituents of the medium were as follows:

- Mannitol 10 g
- Yeast extract 1.0 g
- NaCl 0.1 g
- K₂HPO₄ 0.5 g
- MgSO₄ 7H₂O 0.2 g
- Agar agar 20 g
- Distilled water 1000 g
- Congo red 2.5 ml of 1% solution

One ml of the dilution was added to each petriplate containing 15 ml of CRYMA medium. The petriplates were incubated at 30°C±2 for one week. Distinct white, translucent, glistening elevated colonies of Rhizobium sp. which developed on the media in the petriplates were picked up and purified by reculturing.
Pure culture of *Rhizobium* sp.

Yeast extract mannitol agar (YMA) was used for pure culturing of *Rhizobium* sp. (Fred *et al.*, 1932). The composition of YMA used was as follows:

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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</thead>
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<tr>
<td>K$_2$HPO$_4$</td>
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</tr>
<tr>
<td>Mannitol</td>
<td>10 g</td>
</tr>
<tr>
<td>MgSO$_4$·7H$_2$O</td>
<td>0.2 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>0.1 g</td>
</tr>
<tr>
<td>Yeast extract</td>
<td>0.4 g</td>
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<tr>
<td>Agar-agar</td>
<td>15 g</td>
</tr>
<tr>
<td>Distilled water</td>
<td>100 ml</td>
</tr>
<tr>
<td>pH</td>
<td>6.8-7.0</td>
</tr>
</tbody>
</table>

The medium was autoclaved at 15 lb p.s.i. (120°C) for 20 minutes. The medium was poured in sterilized petriplates. After solidification of the medium the tested *Rhizobium* sp. isolate from the black gram was inoculated in the plates in aseptic conditions at laminar flow bench. After inoculation, the petriplates were kept at 30°C (±2) in an incubator for one week after which colonies developed in the plates. The bacterium was transferred in culture tubes containing YMA medium.

**Soil based culture**

For artificial inoculation of black gram, the soil based culture of *Rhizobium* sp. was prepared and seed dressing was done prior to sowing the seeds according to the treatments.

For culturing root nodule bacterium in soil, a mixture of field soil and compost in the ratio of 1:1 was prepared. One Kg of the soil-compost mixture was autoclaved and the pH was maintained at 7 by mixing 10 g CaCO$_3$. After that 10 g sugar (commercial) and 0.5 g K$_2$HPO$_4$ were added to the soil-compost mixture. Then pure culture
Rhizobium sp. grown on YMA was mixed thoroughly in the mixture. This mixture of Rhizobium sp. soil and compost was used for inoculating the seeds of black gram.

Inoculation with root nodule bacterium

Inoculation of the seeds of black gram was done prior to their sowing by using soil-based culture of Rhizobium sp. Commercial sugar and water were added to the soil-based culture with thorough mixing. The seeds were treated with this mixture followed by drying the seeds in shade for about half an hour before sowing.

Plant culture and treatment

For studying the effects of SO₂ and O₃ seeds of the test plant, black gram (Vigna mungo (L.) Hepper) cv. Type 9 Pantnagar were surface sterilized for 2 min. in 0.1% HgCl₂ solution. Seeds were sown in 72 clay pots (9cm diam.) having sterilized field soil (66% sand, 24% silt, 8% clay, 20 M., pH 7.5). The sowing was done in 4 sets. Each set consisted of 18 pots. The first set of pots contained only plants i.e. without any root symbiont, G. caledonicum or Rhizobium (uninoculated). In the second set, plants were raised from seeds, treated Rhizobium (single inoculation) in the third set, plants were inoculated with G. caledonicum (single inoculation). In the fourth set of plants were inoculated with both Rhizobium sp. and G. caledonicum (dual inoculation).

The plants of the experiments, according to the designated treatments, were exposed intermittently to different doses of gaseous of air pollutants, sulphur dioxide (SO₂) and ozone (O₃) in exposure chambers. The following was the pattern of treatments for the two (SO₂ and O₃) air pollutants.

1. Sulphur dioxide

Two concentrations of SO₂ (0.05 and 0.1 ppm) were used for exposure of the plants designated to receive the SO₂ treatments.
I. Sulphur dioxide

(a) Control set

Plant (uninoculated)
Plant + Rhizobium sp. (single inoculation)
Plant + G. caledonicum (single inoculation)
Plant + G. caledonicum + Rhizobium sp. (dual inoculation)

(b) Exposed set

(i) Plant + 0.05 ppm SO2
Plant + Rhizobium sp. + 0.05 ppm SO2
Plant + G. caledonicum + 0.05 ppm SO2
Plant + G. caledonicum + Rhizobium sp. + 0.05 ppm SO2

(ii) Plant + 0.1 ppm SO2
Plant + Rhizobium sp + 0.1 ppm SO2
Plant + G. caledonicum + 0.1 ppm SO2
Plant + G. caledonicum + Rhizobium sp + 0.1 ppm SO2

Plant of set (i) and (ii) were exposed for 3 h on alternate days.

2. Ozone

Plant were exposed to ozone using two sequences of its concentrations i.e. (i) 0.02-0.05-0.02 ppm O3, (ii) 0.05-0.1-0.05 ppm O3. Exposure durations for each concentration in the both sequences also varied as given below in the treatments.
II. Ozone

(a) Control Set

Plant (uninoculated)

Plant + *Rhizobium* sp. (single inoculation)

Plant + *G. caledonicum* (single inoculation)

Plant + *G. caledonicum* + *Rhizobium* sp. (dual inoculation)

(b) Exposed set

(i) Plant + 0.02-0.05-0.02 ppm O₃

Plant + *Rhizobium* sp. + 0.02-0.05-0.02 ppm O₃

Plant + *G. caledonicum* + 0.02-0.05-0.02 ppm O₃

Plant + *G. caledonicum* + *Rhizobium* sp. + 0.02-0.05-0.02 ppm O₃

Plants were exposed on alternate days. Total exposure was given continuously for 7 h with a change of concentration and duration of exposure i.e. 0.02 ppm for 2 h to start with, followed by 0.05 ppm for 3 h and again 0.02 ppm for 2 h.

(ii) Plant + 0.05-0.1-0.05 ppm O₃

Plant + *Rhizobium* sp. + 0.05-0.1-0.05 ppm O₃

Plant + *G. caledonicum* + 0.05-0.1-0.05 ppm O₃

Plant + *G. caledonicum* + *Rhizobium* sp. + 0.05-0.1-0.05 ppm O₃

Like exposed set (i) exposure was the also given continuously for 7 h with a change of concentration and duration of exposure i.e. 0.05 ppm for 2 h in the beginning, 0.1 ppm for 3 h, and again 0.05 ppm for 2 h.
EXPOSURE SYSTEM

Exposure chamber

The exposure system consisted of 3 chambers each of dimensions 90 x 90 x 120 cm. Two chambers were used for air pollutant exposures and the third one as a control (ambient air). Each chamber was made of transparent glass fibre, with an exhaust duct at the top and double-walled bottom, the upper wall being perforated while the lower wall was equipped with a blower assembly. A fumigation controller regulated the voltage supply to the blower and displayed it on a meter fitted to the chamber. The chambers had a movable front door and were horizontally partitioned by a meshed iron tray to provide additional space for the placement of pots. The exhaust duct (20 x 20 cm) of the exposure chamber were connected to a vertical exhaust pipe fitted in the roof of the glasshouse (Fig. 1).

Gas generation

Sulphur dioxide was generated in a generator which produced SO$_2$ gas by the action of sulphuric acid (H$_2$SO$_4$) on sodium sulphite (Na$_2$SO$_3$) under control reaction conditions. The amount of Na$_2$SO$_3$ and H$_2$SO$_4$ discharged from the reagent bottles mounted over the SO$_2$ generator were determined by collecting the solution dropping through capillary tube in a graduated cylinder for sometime and expressing the rate in ml/min. On the basis of flow rate or solution feeding rate, solutions of Na$_2$SO$_3$ and H$_2$SO$_4$ (10%) was prepared to produce required amount of SO$_2$ gas/min. On complete reaction 1M Na$_2$SO$_3$ produces 1 SO$_2$ or 126 mg Na$_2$SO$_3$ produces 64 mg SO$_2$.

$$\text{Na}_2\text{SO}_3 + \text{H}_2\text{SO}_4 \longrightarrow \text{SO}_2 + \text{Na}_2\text{SO}_4 + \text{H}_2\text{O}$$

Ozone

Ozone was generated by subjecting dry oxygen to the action of silent electric discharge in an apparatus called ozoniser

$$3\text{SO}_2 \leftrightarrow 2\text{O}_3$$

The concentration of SO$_2$ (0.05 ppm and 0.1 ppm) and
FIGURE 1
EXPOSURE CHAMBER
O₃ (0.02 ppm, 0.02-0.05-0.02 ppm and 0.05-0.1-0.05 ppm) was also determined by sampling the air using a portable air sampler (Kimoto Electricals, Japan) and was analysed by spectrophotometry (Anon., 1986). The outlet of the gas generator was connected to the gas inlet nozzles provided in the blower housing of the exposure chamber.

**Treatment with the gaseous air pollutants (SO₂ and O₃)**

**Treatment with SO₂**

All set of black gram (22-day-old plants) designated to be treated with SO₂ were exposed for 3 h on alternate days to 0.05 ppm and 0.1 ppm till the termination of the experiment (80 days). Total 29 exposures were made in the experiment. After every exposure, pots were again transferred to glasshouse.

**Treatment with O₃**

Twenty two-day-old plants of black gram of the exposed sets were treated with O₃. Plants were exposed to two concentration sequences 0.02-0.05-0.02 ppm and 0.05-0.1-0.05 ppm on alternate days. The exposures were continued for 7 h with a change of concentration and duration of exposure i.e. (1) 0.02 ppm for 2 h, 0.05 ppm for 3 h and again to 0.02 ppm for 2 h (2) 0.05 ppm for 2 h, 0.1 for 3 h and again 0.05 ppm for 2 h.

All sets were exposed, according to the schedule described above, both for SO₂ and O₃ till the termination of experiment (80 days). Total 29 exposures for each concentration were made for the crop. After every exposure, pots were again transferred to glasshouse. In both the experiments, each treatment was replicated six times and pots were arranged in complete randomized block design (CRBD) in the glasshouse.

**Fly ash**

For studying the effect of amendment of soil with fly ash, clay pots of 9 cm diam. were filled with a mixture of soil and fly ash and autoclaved. Fly ash for the experiment was collected from a thermal power plant located at Kasimpur, 15 Km away from the Aligarh Muslim
University Campus. The power plant uses bituminous type of coal. Surface sterilized seeds of black gram were sown in sterilized clay pots containing a mixture of soil and fly ash in different ratios (V/V). Different levels (V/V) of fly ash used in the study (20,40,60,80,100%) were obtained by adding fly ash to the sandy loam field soil (66% sand, 24% silt; 8% clay, O.M 2%, pH 7.5) in desired amounts. Pots were divided into four sets each having 36 pots. In the first set of pots, the plants were kept uninoculated (control). Inoculation of symbionts (singly and in combination) were done in 3 sets. Inoculation with *Rhizobium* sp. (seed dressings) and *G. caledonicum* (1000 spores/pot) were done in same manner as described earlier.

In the experiment with fly ash following were the treatments.

(a) Unamended soil (0% fly ash)

- Plant (uninoculated)
- Plant + *Rhizobium* sp.
- Plant + *G. caledonicum*.
- Plant + *Rhizobium* sp. + *G. caledonicum*.

(b) Amended soil (fly ash level)

- Plant + 20% fly ash
- Plant + *Rhizobium* sp. + 20% fly ash
- Plant + *G. caledonicum* + 20% fly ash
- Plant + *Rhizobium* sp. + *G. caledonicum* + 20% fly ash
- Plant + 40% fly ash
- Plant + *Rhizobium* sp. + 40% fly ash
- Plant + *G. caledonicum* + 40% fly ash
- Plant + *Rhizobium* sp. + *G. caledonicum* + 40% fly ash
- Plant + 60% fly ash
Plant + *Rhizobium* sp. + 60% fly ash

Plant + *G. caledonicum* + 60% fly ash

Plant + *Rhizobium* sp. + *G. caledonicum* + 60% fly ash

Plant + 80% fly ash

Plant + *Rhizobium* sp. + 80% fly ash

Plant + *G. caledonicum* + 80% fly ash

Plant + *Rhizobium* sp. + *G. caledonicum* + 80% fly ash

Plant + 100% fly ash

Plant + *Rhizobium* sp. + 100% fly ash

Plant + *G. caledonicum* + 100% fly ash

Plant + *Rhizobium* sp. + *G. caledonicum* + 100% fly ash

Each treatment was replicated six times and pots were arranged in complete randomised block design (CRBD) on glasshouse benches.

**Parameters**

After termination of the experiments, following parameters were determined for each treatment of the experiments.

Root and shoot lengths

Fresh and dry weight of shoot and root

Yield (number of pods per plant and number of seeds per pod)

Root colonization by the VAM fungus

Number of VAM spores/100 g of soil

Number and dry weight of nodules/root system

Chlorophyll content of leaf
Nitrogen and phosphorus content of roots and shoots

Protein content of seeds.

Plant growth and yield

After termination of the experiment, for determining length and fresh weight of shoot and root, plants of each treatment were taken out from the pots and soil particles adhering of roots were removed with tap water and properly labelled and brought to the laboratory. In the laboratory, lengths of shoot and root were measured by measuring tape and fresh weights of shoot and root were determined by physical balance. For determining dry weights of root and shoot, plants from each treatment were wrapped in a blotting sheet, labelled and dried in a hot air oven at 60°C for 24 h and weighed. Number of pods/plant and number of seeds/pod were counted before dry weight was taken, to determine the yield in each treatment.

Root colonization by the VAM fungus and estimation of VAM spores from the soil

At the termination of the experiments, root colonization of the plants by the VAM fungus was assessed. Three soil cores were taken with the help of an auger (2.5 cm diam.) from each pot of the treatments inoculated with G. caledonicum. The three soil cores from each pot of each treatment were mixed which was considered as one sample. Soil of each sample was suspended in water and roots were retained on a 100 mesh sieve. Roots were cut 1 cm long. Root pieces (1 cm long) were cleared by 10% KOH and alkaline H₂O₂ and stained by Trypan blue (0.05% in lactophenol) solution according to the procedure given by Phillips and Hayman (1970).

Percentage of root colonization was determined by slide method (Giovanetti and Mosse, 1980). Ten root samples were mounted on each glass slide, selected randomly from stained samples and examined microscopically (25x) for root colonization. One hundred to one hundred and fifty (100-150) root segments from each sample were used for the assessment. The presence or absence of colonization in each root segment was recorded and result was expressed as
percentage of root colonized. The root colonization (mycorrhizal infection in the roots) was calculated as follows:

\[
\text{VAM association} = \frac{\text{No. of mycorrhizal segments}}{\text{Total no. of segments examined}} \times 100
\]

**Estimation of spores**

For estimating the spores of *G. caledonicum* in the same soil samples used for assessment of root colonization, spores were isolated from the soil of the treatments inoculated with VAM fungus by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Repeated washings with Ringer's solution were done to separate the spores from soil matter. The suspension thus obtained was made upto 50 ml. The spores were counted in 1 ml of suspension in a nematode counting dish under the stereoscopic microscope. The final number of spores/100 g soil was calculated.

**Root nodulation**

Before drying the plants for determining their dry weight, number of nodules per root system in the treatments having *Rhizobium* sp. inoculation were counted. Then after nodules were detached from the roots and placed separately for each treatment in labelled petridishes. The petridishes containing the nodules were kept in an hot air oven at 60°C and dry weight of the nodules were determined. This dry weight was added to the root dry weight of respective treatments.

**Plant analysis**

Chlorophyll content of leaves, nitrogen and phosphorus contents of root and shoot, protein content of seeds were estimated from the plants of each treatment.

**Chlorophyll estimation**

Chlorophyll content of leaves of black gram from different treatments of the experiment was estimated. Leaves of different ages (tender to old leaves) were collected from all the sets of black gram plants of each treatment. One gram of interveinal region of the leaves was ground in 40 ml of 80% acetone with the help of mortar and pestle.
The suspension was decanted in buchner funnel having two Whatman paper no.1. The filtration was done with the help of suction pump. The residue was ground thrice adding with 30, 20 and 10 ml of acetone respectively. The suspension was decanted in buchner funnel and filtered in vacuum. At last mortar and pestle were rinsed with 80% acetone, transferred in buchner funnel and filtered in vacuum. The filtrate were transferred in 100 ml volumetric flask and the volume was made upto capacity. The transmittance were read at 645,663 and 635 nm at spectrophotometer. The chlorophyll a, b and total chlorophyll were calculated accordingly by using optical density (O.D.) i.e. by using % transmittance (Machinney,1941).

\[
\text{Chl.a in fresh tissue}=12.7(\text{O.D.663})-20.6(\text{O.D.645}) \times \frac{1000W}{1000W} \\
\text{Chl.b in fresh tissue}=22.9(\text{O.D.645})-4.68 (\text{O.D.663}) \times \frac{1000W}{1000W} \\
\text{Total chl.in fresh tissue}=20.2 (\text{O.D. 645})+8.02(\text{O.D. 663}) \times \frac{1000W}{1000W}
\]

**Nitrogen and phosphorus estimation from shoot and root**

For estimation of nitrogen and phosphorus, shoot and root samples were digested as given below:

**Digestion of shoot and root samples**

Shoot and root samples of plants from various treatments of the experiments were digested first according to the following method.

100 mg of oven dried shoot and root powder were transferred in 50 ml kjeldahl flask, then 2 ml of chemically pure H₂SO₄ was added and flasks were heated on kjeldahl assembly for about 2 h, till the dense fume had given-off and the contents had turned black. The 0.5ml of pure 30% H₂O₂ was added after 15 minutes of cooling. Heating was done again till the colour was changed into light yellow. It was heated again for half an hour and flasks were cooled for 10 min for getting the extract clear. Then 3-4 drops of 30% H₂O₂ were added dropwise
followed by heating for 15 min. After that digested material was transferred in 100 ml volumetric flask with 3-4 washing and used for estimating N and P etc. present in the shoots and roots (Linder, 1944; Lundegardh, 1951).

**Nitrogen from shoot and root**

Prior to estimating the N content present in the digested material of shoot and root, standard curve was drawn by the following procedure:

0.236 g of ammonium sulphate was dissolved in 100 ml of solution, then 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0 ml solutions were poured in test tubes respectively. The volume was made up to 5 ml in each test tube by adding distilled water. A control was also run side by side. After that 0.5 ml Nesseler's reagent was added followed by 5 ml of distilled water. The percentage transmittance was read at 525 nm on spectrophotometer on developing yellow organe colour after half an hour. Then a curve was drawn on graph between concentration and O.D.

**Estimation**

10 ml of aliquot (digested shoot and root material) was taken in 100 ml volumetric flask and 2 ml, 2.5 N NaOH was added to neutralise the excess amount of acid present. Then 1 ml of 10% sodium silicate was added to prevent turbidity and volume was made up to capacity. 5 ml of aliquot was taken in 3 test tubes followed by addition of 0.5 ml of Nesseler's reagent with shaking. Then 10 ml volume was made by adding distilled water. After waiting for 5 min the % transmittance was read at 525 nm. Concentrations were read from standard graph by using O.D. (Linder, 1944).

**Phosphorus**

At first a standard curve was prepared. Different concentrations of KH$_2$PO$_4$ solution ranging from 0.1 to 1 ml were taken in 10 separate test tubes and the volume of each test tube was maintained up to 5 ml. Then 1 ml ammonium molybdic acid and 0.4 ml of 1 amino-2-nepthol-4-sulphonic acid were added in each test tube
followed by making the volume upto 10 ml with distilled water. After half an hour % transmittance was read at 625nm. Then standard curve was drawn between concentration and O.D.

**Estimation**

5 ml of aliquot (digested shoot and root) were taken in three test tube to which 5 ml of distilled water was added. After that 1 ml of ammonium molybdic acid was added, with shaking, followed by addition of 0.4 ml 1amino-2-nepthol-4-sulphonic acid. The control was also run side by side. Percentage transmittance was read at 625 nm after half an hour. Concentrations were read from standard graph by using O.D. (Fiske and Row, 1925).

**Protein estimation**

The protein content of the seeds of black gram was estimated by the method given by Lowry et al., (1951).

Following reagents were prepared for estimation soluble and insoluble proteins present in the seeds:

- Reagent A-2% sodium carbonate in 0.1 N NaOH in ratio of 1:1.
- Reagent B-0.5% CuSO₄ in 1% sodium tartrate in ratio of 1:1.
- Reagent C-50 ml reagent A+1 ml reagent B (Alkaline CuSO₄). (Carbonate CuSO₄ soln.) .
- Reagent D-50 ml of 2% sodium carbonate + 1 ml reagent B .
- Reagent E-Folin's reagent diluted to make 1N (Diluted Folin's reagent).

**Standard curve**

Before actual estimation, a standard curve was prepared by dissolving 40 mg of egg albumin in 0.1N NaOH solution, the volume of which was made upto 100 ml by adding distilled water. From this solution, aliquots of 0.1 ml to 1 ml were taken in 10 test tubes. Reagent A was now added to the test tubes. After 10 min 0.5 ml reagent E was
added to the test tubes. The % transmittance were read at 660 nm and standard curve was drawn between O.D. and concentration.

Soluble protein

50 mg dry powder of seeds was ground with 5 ml of double distilled water with mortar and pestle. Then water extract was decanted in centrifuge tube for centrifugation at 4000 rpm for 10 min. The supernatant was collected in 50 ml volumetric flask and residue was retained in centrifuge for estimating insoluble proteins. After making the volume upto 50 ml by adding DDW, 1 ml of water extract was transferred in a 10 ml test followed by addition of 5 ml of reagent C. After mixing, solution was left as such for 10 minutes. Then 5 ml of reagent E was added and mixed immediately. The control was run along with experimental set. Percent transmittance was read at 660 nm after half an hour. The corresponding protein content were measured, by using the standard curve.

Insoluble protein

The residue retained in the centrifuge tube was used for insoluble protein estimation. 5ml of 5% tricholoracetic acid was added to the residue with shaking. After half an hour it was centrifuged at 4000 rpm to 10 min. The supernatant was discarded. 5ml of 1N NaOH was added in the residue with vigorous shaking. After half an hour it was again centrifuged and supernatant was collected in 50 ml volumetric flask and volume was made upto 50 ml within 1N NaOH.

1 ml of the solution was taken in test tube with 5 ml of reagent D followed by mixing. After 10 min. 0.5 ml of reagent E was added with immediate mixing. 1N NaOH was used in control. Percent transmittance were read at 660 nm after 30 min. The protein content was calculated by using the standard curve. By adding soluble and insoluble protein, total protein was calculated.

Statistical Analysis

Experiments were conducted in complete randomized block design, but while analysing the data, this was further extended to split the factors and analysed by extending the method adopted by Panse and
Sukhatme (1954). In the experiment, black gram was selected as factor one and symbiont treatments (pollutant, *Rhizobium* sp., *G. caledonicum*) as factor two. The data obtained were subjected to analysis of variance (ANOVA) to determine significance and C.D. was calculated at $P=0.05$ to separate the means of replicates for significance. The ANOVA model adopted for the analysis of variance comes as follows, where

- $R$ = Replicates
- $F_1$ = Factor one
- $F_2$ = Factor two
- $d F$ = Degree of freedom
- $SS$ = Sum of squares
- $MS$ = Mean of squares
- $F_{\text{cal.}}$ = $F$ value calculated
- $F_{\text{Tab.}}$ = $F$ value tabulated

### ANOVA Table

<table>
<thead>
<tr>
<th>Variable</th>
<th>d.F.</th>
<th>SS</th>
<th>MS</th>
<th>$F_{\text{cal}}$</th>
<th>$F_{\text{Tab}}$</th>
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<tbody>
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<td>$R-1$</td>
<td>Calculated as per procedure</td>
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<td>$RMS/EMS$</td>
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<td>$F_1$</td>
<td>$(F_1-1)$</td>
<td>$SS/F_1dF$</td>
<td>$F_1MS/EMS$</td>
<td>$F_1dFVsEdF$</td>
<td></td>
</tr>
<tr>
<td>$F_2$</td>
<td>$(F_2-1)$</td>
<td>$SS/F_2dF$</td>
<td>$F_2MS/EMS$</td>
<td>$F_2dFVsEdF$</td>
<td></td>
</tr>
<tr>
<td>Interaction</td>
<td>$(F_1-1)(F_1-1)$</td>
<td>$SS/F_1xF_2dF$</td>
<td>$F_1xF_2MS/EMS$</td>
<td>$F_1/F_2dFVsEdF$</td>
<td></td>
</tr>
<tr>
<td>i.e. $F_1 \times F_2$</td>
<td>$(F_2-1)$</td>
<td>[\begin{align*} &amp;SS/F_1xF_2dF \ &amp;F_1xF_2MS/EMS \end{align*}]</td>
<td>$F_1xF_2MS/EMS$</td>
<td>$F_1/F_2dFVsEdF$</td>
<td></td>
</tr>
<tr>
<td>Error</td>
<td>$(R-1)(F_1-1)+(R-1)F_1(F_2-1)$</td>
<td>$SS/EdF$</td>
<td>$F_1xF_2MS/EMS$</td>
<td>$F_1/F_2dFVsEdF$</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>$(RxF_1xF_2)-1$</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


Standard error (SE) was calculated as follows prior to calculation of the CD. (Critical difference).

\[
\text{SE for } F_1 = \sqrt{\frac{2\text{EMS}}{RXF_2}}
\]

\[
\text{SE for } F_2 = \sqrt{\frac{2\text{EMS}}{RXF_1}}
\]

\[
\text{SE for } F_1 \times F_2 = \sqrt{\frac{2\text{EMS}}{R}}
\]

CD was calculated at P=0.05 with the help of calculated S.E. as follows.

\[
\text{CD for } F_1 = \text{SE for } F_1 \times \text{t value at 5%}
\]

\[
\text{CD for } F_2 = \text{SE for } F_2 \times \text{t value at 5%}
\]

\[
\text{CD for } F_1 \times F_2 = \text{S.E. for } F_1 \times F_2 \times \text{t value at 5%}
\]

In this way, three CD were calculated, significance and non-significance of CD was calculated with help of ANOVA table if F cal was found to be greater than F. Tab., the data were considered as significant and a vice-versa.