CHAPTER 2

MATERIALS AND METHODS
2. MATERIALS AND METHODS

2.1 Plant material

Four varieties of fenugreek were used for the current study. Seeds of *Trigonella foenum-graecum* (fenugreek) varieties: *Pusa early bunching* (PE), *Pusa Kasuri Methi* (KM) – *(Trigonella coriculata)*, *Hissar sonali* (HS) were obtained from IARI, New Delhi and Local variety (LV); obtained from the local market.

2.2 Experimental Design for UV exclusion experiment and growth condition

All the field experiments were conducted in the Botanical garden of School of Life Sciences, Devi Ahilya University, Indore, M.P., (Indore Location is at 22°43’ north latitude and 75°49’60” east longitude) India. Seeds of *T. foenum-graecum* were treated with recommended fungicides viz. bavistin and dithane M at 2 g/kg seeds. The experiments were carried out during favourable season October to February each year when the average daily solar UV-B dose was approximately 50% higher than the average daily dose received in the temperate region. Seeds were surface sterilized and then inoculated with a slurry of *Rhizobium japonicum* strain before sowing and sown in polythene bags containing a mixture of coarse sand, black soil and yard manure inside iron cages (4 feet L x 3 feet W x 4 feet H). The cages were wrapped with UV-B, and UV-B+A cut off filters (Garware polyesters Ltd., Mumbai) that excluded either UV-B (< 315 nm) or UV-B+A (< 400 nm) and the control plants were grown under an ordinary polythene filter permissible to UV (280-400 nm) radiation (Fig 2.1 A & B) or under cages without any filter. The transmission characteristics of the filters measured by Shimadzu (UV-1601) spectrophotometer are as given in Fig. 2.2. (Kataria and Guruprasad, 2012a, b). The transmission characteristics of filters did not change during the experimental period and these filters did not emit fluorescence in visible regions. Filters were changed after every 20 days. Open control seedlings were exposed to ambient solar UV radiation from the time of germination. Metal cages received full solar radiation during the day without any shading. For study of roots and nodule characteristics plants were grown in polythene bags. There was no significant temperature difference between filter control and UV excluded chambers as horizontal holes in the chamber allowed passive air ventilation. The
experiments were conducted in a randomized block design with three replicates for each treatment.

**Fig. 2.1:** Experimental setup for growing plants under exclusion of UV-B and UV-B+A
2.3 Radiation measurements

Absolute solar irradiance with or without UV-B or UV-B+A was measured using a radiometer (Solar light PMA2100, Glenside, PA., U.S.A). The ambient solar irradiance during experimental period at midday was 1450 µmol m\(^{-2}\) s\(^{-1}\), the loss in light intensity at midday by 11.8% (1280 µmol m\(^{-2}\) s\(^{-1}\)) under -UV B+A filters and -UV-B filters was 12.5% (1270 µmol m\(^{-2}\) s\(^{-1}\)) and 4.2% (1390 µmol m\(^{-2}\) s\(^{-1}\)), under polythene filter transmissible to UV (filter control). The PAR intensity for normal plant growth was observed to be optimal saturating light.
2.4 Growth Analysis:

2.4.1 Growth parameters above ground parts

**Plant height:** Measurements of plant height were taken at the 30\textsuperscript{th} DAE, 45\textsuperscript{th} DAE and at 60\textsuperscript{th} DAE in three replicates of five plants each. Plant height was measured from the soil line to shoot tip. Plant height was measured by placing the plant on a centimeter scale.

**Leaf area:** Leaf area was measured by using Leaf Area Meter CI-202 (CID Bio Sciences, USA).

**Fresh weight and Dry weight** Measurements of Fresh weight and Dry weight were taken at the 30\textsuperscript{th} DAE, 45\textsuperscript{th} DAE and at 60\textsuperscript{th} DAE in randomly selected three replicates of five plants each and the data were recorded. Total plant fresh weight was taken after removing the plants and washing roots thoroughly with water. To obtain dry matter, plants were dried at 60\textdegree C for 72 hours and weighed on an analytical balance.

2.4.2 Growth Analysis: Below ground parts

Plants were grown for 90 DAE (Days after emergence) under UV-B and UV-B+A excluded conditions. Fifteen plants from each treatment and control were carefully uprooted from the soil at 15\textsuperscript{th} DAE, 30\textsuperscript{th} DAE, 45\textsuperscript{th} DAE and at 60\textsuperscript{th} DAE, to record root length (length of longest root).

**Root fresh and dry weight**

Plant roots with nodules at each sampling were washed and dried on filter paper and weighed for the fresh weight. For dry weight roots were dried at 60\textdegree C for 72 hours and weighed.

**Number of root nodules/plant**

Nodules on each root were counted carefully and recorded per plant.

**Nodule fresh weight**
Nodules were taken out at each sampling, washed and dried on filter paper. Weight was recorded in gm/plant for all treatments.

2.4.3 Plant yield and components of yield

Yield parameters including number of pods/plant, seed weight/plant and 100 seed weight have been taken at the crop maturity in three replicates of five plants each. Pods were separated and the total number of pods were recorded. Dry weights of seeds were recorded after drying at 35–40°C for 10 days.

2.4.4 Harvest index

Harvest index was estimated as the ratio of seed yield to total dry matter yield, calculated according to Moosavi et al., (2011). The harvest index (HI) increase has been used as a simple means to analyze and predict crop yield in experiment (mean of ten plants).

\[
\text{Harvest Index} (\%) = \frac{\text{Seed weight of selected 10 plants}}{\text{Biological weight of selected 10 plants}} \times 100
\]

2.4.5 UV Sensitivity Index

Differences in the UV-sensitivity of the four varieties were ascertained by a UV sensitivity index (UV-SI) for UV-B and UV-B+A calculated according to the following equation (Saile-Mark and Tevin, 1997):

\[
\text{UV B-SI} = \frac{PH - (UV B)}{PH - (UV B')} \times \frac{DW - (UV B)}{DW - (UV B')} \times \frac{SY - (UV B)}{SY - (UV B')}
\]

\[
\text{UV A/B-SI} = \frac{PH - (UV A / B)}{PH - (UV A / B')} \times \frac{DW - (UV A / B)}{DW - (UV A / B')} \times \frac{GY - (UV A / B)}{GY - (UV A / B')}
\]

2.5 Reagents and Chemicals:

The following chemicals were purchased from the respective companies shown:

**Sigma chemical Co., USA:** Bovine serum albumin (BSA), Superoxide dismutase (SOD), Pyrogallol.
**Merck, Germany:**- Trichloroacetic acid (TCA), Riboflavin, Pyridine.

**Loba Chemie, Bombay, India:** - Guaiacol.

**S.D Fine chemicals, Bombay, India:** - Hydrogen peroxide, Sodium phosphate, potassium phosphate, Folin phenol.

**Hi Media, Bombay, India**: - 2,2'-bipyridyl, Dithiothreitol (DTT), N-ethylmaleimide (NEM), FeCl₃, L-ascorbic acid, Polyvinyl pyrolidone (PVP) NADPH, glutathione (oxidized form), Sodium potassium tartrate, EDTA.

**Qualigens**: – HCl, H₂SO₄, Alcohol

**Laser Gases, New Delhi, India**: - Ethylene standard, Acetylene.

### 2.6 UV Absorbing Substances Measurement

Accumulation of UV absorbing substances (UAS) in fully expanded trifoliate leaves was measured at the 30th DAE (5th node), 45th DAE (7th node) and at 60th DAE (10th node) respectively. UAS was determined spectrophotometrically from acidified methanol extract by the method of Mazza et al., (1999). For spectrophotometric determination, 0.50 cm diameter leaf disc was placed in 5 mL of 99:1 (methanol:HCl) and allowed to extract for 48 h at 4°C. Absorbance of the extracts was read at 305 nm for determination of total UAS. Absorbance was expressed on leaf fresh weight basis (A mg⁻¹ fw).

### 2.7 Chlorophyll content

The total chlorophyll and carotenoids content was determined in (fully opened matured leaves of 7th node (at 45 DAE) by dimethyl sulfoxide (DMSO) method (Hiscox and Israelstam, 1979). 50 mg of fresh tissue was kept dipped in 5ml of DMSO overnight at room temperature. The absorbance was then taken at 480, 649 and 665nm with Shimadzu UV/VIS 1601 spectrophotometer. Equations of Wellburn (1994) were used to calculate the chl a, chl b, total chl and carotenoids concentrations.

\[
\text{Chl } a \hspace{1cm} (\text{mg/l}) = (12.19 \times A_{665}) - (3.45 \times A_{649})
\]
Chl b \ (mg/l) = (21.99 \times A_{649}) - (5.32 \times A_{665})

Chl t \ (mg/l) = (7.18 \times A_{665}) + (17.32 \times A_{649})

Crt \ (mg/l) = (A_{480} \times 1000 - 2.14 \times Chl a - 70.16 \times Chl b) / 220

Chl a = Chlorophyll a

Chl b = Chlorophyll b

Chl t = Total Chlorophyll

Crt = Carotenoids.

2.8 Determination of net photosynthetic rate, transpiration rate and stomatal conductance and gas exchange by Infra Red Gas Analyzer (IRGA)

Photosynthetic parameters like rate of photosynthesis (µmol CO₂ m⁻² s⁻¹), internal CO₂ concentration (µmol CO₂ mol⁻¹), stomatal conductance (mol H₂O m⁻² s⁻¹) and transpiration rate (mmol H₂O m⁻² s⁻¹) of the leaves of (fully opened matured leaves of 7th node at 45 DAE) were measured by a portable photosynthetic system (Li-6200, LI-COR Inc., Lincoln, Nebraska, Serial No. PPS 1332 USA). Measurement was done in intact plants grown in natural sunlight or under UV exclusion filters in field conditions, under ambient temperature and CO₂ concentration. On clear days at noon photosynthetic photon flux density (PPFD) was 1,300–1,600 µmol m⁻² s⁻¹, air flow (500 µmol s⁻¹), and CO₂ concentration (350–380 ppm). Before recording the measurement, the IRGA was calibrated and zero was adjusted approximately every 30 min during the measurement period.

2.9 Fluorescence measurements

Chlorophyll a (Chl a) fluorescence transients exhibited by dark-adapted (30 min) (fully opened matured leaves of 7th node at 45 DAE) was measured using a Handy PEA fluorimeter (Plant Efficiency Analyzer, Hansatech Instruments, King’s
Lynn, Norfolk, UK). The transients were induced by red light (peak at 650 nm) of 600 Wm$^2$ (3,200 µE m$^{-2}$ s$^{-1}$) provided by an array of six light-emitting diodes focused on a 4 mm diameter spot on the leaf surface to provide homogenous illumination over the exposed area of the sample. Data were recorded for 1 s with 12-bit resolution; the data acquisition was every 10 µs for the first 2 ms and every 1 ms thereafter (Strasser et al., 1995). All the measurements were recorded at 25 ± 1°C. The Chl $a$ fluorescence transients recorded, when plotted on a logarithmic scale, clearly showed polyphasic fluorescence rise kinetics (O–J–I–P phases). The fluorescence intensity at 20 µs was considered as the intensity $F_0$ (O phase) when all reaction centers are opened, the fluorescence intensity at 2 ms was the J phase, 30 ms was the I phase, and the maximum fluorescence ($F_m$) was the P phase ($F_p$ equals here to $F_m$) since the excitation intensity is high enough to ensure the closure of all reaction centers of photosystem II (PSII). OJIP transient was analysed according to the JIP test and the following parameters were calculated: (1) maximum quantum yield of primary photochemistry ($F_v/F_m$); (2) the efficiency by which a trapped excitation, having triggered the reduction of QA to QA$^-$, can move an electron further than QA$^-$ into intersystem electron transport chain ($\psi_o= ET0/CS_m$); (3) the quantum yield of electron transport ($\varphi_Eo=ET0/ ABS$) and (4) performance index (PI) reflecting the performance of the overall energy flow. These parameters were calculated as described by Strasser et al. (2000; 2004) using the software ‘Biolyzer HP 3’ (the chlorophyll fluorescence analysing program by Bioenergetics Laboratory, University of Geneva, Switzerland).

2.10 Biochemical estimations

All nodule parameters and biochemical estimations like nitrogenase and leghemoglobin content were done at 45 DAE, since nodulation was at its peak level at this age.

2.10.1. Nitrogenase activity

Nitrogenase (EC 1.18.6.1) activity was determined in 45 DAE plants by the acetylene reduction assay (ARA) (Hardy et al., 1973). Three roots with intact nodules were excised and incubated in a 25-ml incubation vessel sealed with a
flanged rubber septum. 5 ml of the air was withdrawn from the incubation vessel (via the rubber septum in the cap with a syringe) and replaced with an equal volume of \( \text{C}_2\text{H}_2 \). After 60 min incubation at 20°C, 2 ml gas sample was withdrawn and analyzed by gas chromatography (DANI make, DPC-100, Italy) for ethylene formation. The incubation vessel was filled with water to measure the volume occupied by the root (air space). Nodulated roots were removed from the incubation vessel, fresh weight of root with the nodules were taken after blotting. Nodule fresh weight was also taken separately after blotting. Standard curve for ethylene was developed for analysis. Nitrogenase activity was calculated for the linear phase of reaction and expressed as

\[
\text{Nitrogenase activity} = \text{nmol C}_2\text{H}_4 \text{ produced/g fresh weight of root nodule/h.}
\]

The gas samples were then analyzed by gas chromatography.

**Source of Ethylene**

Ethylene gas used as a standard was obtained from Laser gases, Chhattarpur, New Delhi, India.

**Mixture composition concentration of the standard**

<table>
<thead>
<tr>
<th>Components</th>
<th>Ordered</th>
<th>Actual</th>
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<tr>
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<td>1014 ppm</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>BALANCE</td>
<td>BALANCE</td>
</tr>
</tbody>
</table>

Settled pressure – 20 kg/cm²

**2.10.2. Extraction and estimation of leghemoglobin (Lb) content**

Leghemoglobin (Lb) was extracted from the root nodules of 45 DAE plants and measured by the method of Jun et al., (1994). Root nodules (3 g) from the 45 DAE old plants grown under ambient UV radiation, or under exclusion of UV-B and UV-B+A were crushed in liquid nitrogen in a mortar with pestle. The resulting
powder was resuspended in 25 ml of 50 mM sodium phosphate buffer (pH 7.5) containing 1 mM EDTA, 1 mM PMSF, β-mercaptoethanol and 10% polyvinyl pyrrolidone (PVP). The resulting solution was filtered through cheese cloth and centrifuged at 20,000 g for 20 min at 4°C. The deep red supernatant was saturated to 50% with solid (NH₄)₂SO₄ and then centrifuged at 15,000 g for 20 min at 4°C. The pellet was discarded and the red supernatant was saturated to 90% with solid (NH₄)₂SO₄ and then centrifuged at 15,000 g for 20 min at 4°C. The red pellet was resuspended in 15 ml of 20 mM Tris HCl (pH 8.0) containing 1 mM (NH₄)₂SO₄. The absorbance of Lb-containing fraction was detected at 410 nm by using a UV-visible Spectrophotometer. Total soluble protein content was measured in LH-containing fractions by the method of Lowry et al., (1951).

2.10.3. Heme concentration

Heme concentration in leghemoglobin was measured by pyridine hemechromogen assay as described by Appleby and Bergersen (1980). Fresh root nodules (1 mg) were mixed with 10 ml of 50 mM phosphate buffer (6.5 pH) and homogenized. The contents were filtered through two layers of cheesecloth. Nodule debris was discarded and remaining brown filtrate was centrifuged at 20,000 g for 20 min. To 5 ml of extract, 5 ml alkaline pyridine reagent was added and mixed. The resulting hemechrome was equally divided into two portions. To one portion (5 ml) few crystals of sodium dithionate was added to reduce the hemechrome. To the other portion (5 ml), 5 mM of potassium hexacyanoferrate (III) was added to oxidize the hemechrome and the contents of both the test tubes were measured at 556 nm and 539 nm respectively.
2.10.4 Determination of nitrate reductase (NR) activity

Nitrate reductase (E.C. 1.6.6.1) activity in the leaf was determined by the intact tissue assay method of Jaworski (1971). Chopped leaf pieces (100 mg) were incubated for 2 h at 30°C in a 10 mL reaction mixture, which contained 2.5 mL of 0.1 M phosphate buffer, 0.5 mL of 0.2 M potassium nitrate, and 2.5 mL of 5% isopropanol. The nitrite formed subsequently was determined at 540 nm after azocoupling with sulphanilamide and naphthylenediamine dihydrochloride. The NR activity was expressed as nM NO$_2$ g$^{-1}$ FWh$^{-1}$. The amount of nitrite produced was calculated from the standard curve with 7.5 to 200 nM nitrite/ml (Fig. 2.3)

![Figure. 2.3 Calibration curve for nitrite](image_url)
2.10.5: Protein Analysis

Protein was estimated in (fully opened matured leaves of 7\textsuperscript{th} node at 45 DAE) of pusa early bunching by the method described by Lowry et al., (1951). Extracted protein (1ml) was precipitated using 1ml of 20\% TCA and kept for 24 hrs at 4\(^o\)C and centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded and pellet was suspended in 1ml 0.1 N NaOH.

Reagents Used:

Reagent A: 2\% Sodium Carbonate in 0.1 N NaOH.

Reagent B: 0.5\% Copper Sulphate in 1\% Sodium potassium tartarate.

Reagent C: 2ml of reagent B + 100ml reagent A.

Reagent D: 1:1 N Folin Phenol Reagent.

Figure 2.4 Calibration curve for Protein.
**Assay**

4ml of reagent C was added in the reaction mixture consisting of 1ml of protein extract, kept at room temperature for 20 minutes, 0.4 ml of reagent D was subsequently added. After incubation for 30 minutes absorbance was read at 660 nm in a Shimadzu spectrophotometer. A calibration curve was made using Bovine Serum Albumin (BSA) as standard (Fig. 2.4).

**2.11: Extraction and Estimation of the Antioxidant Enzymes:**

**2.11.1: Guaiacol Peroxidase [EC 1.11.1.7]**

Extraction: 100 mg leaf tissues were crushed in a prechilled mortar and pestle using chilled 80 % acetone at 4°C. The extract was centrifuged at 5000 rpm for 10 min. The supernatant was discarded and the pellet redissolved in 10 ml of 0.02 M phosphate buffer (pH 6.4) and centrifuged for 15 min at 10,000 rpm. The buffered supernatant was used for the cytosolic peroxidase assay.

Assay: Peroxidase was assayed by the method of Maehly, (1955). The reaction mixture contained 0.5 ml enzyme extract, 1 ml 20 mM guaiacol and 3 ml 0.02 M phosphate buffer. The reaction was started by the addition of 0.03 ml of \textit{H}_2\textit{O}_2 (1 volume). The initial and final absorbance was noted at 475 nm for 2 min. Activity was calculated as change in OD/ min / mg protein.

**2.11.2: Ascorbic acid peroxidase [EC 1.11.1.11]**

Extraction: 100 mg leaf tissues were crushed in prechilled mortar and pestle in an extraction media containing 50 mM sodium phosphate buffer (pH 7.4), 1 mM EDTA, 1 % PVP and 1 mM ascorbic acid. The homogenate was centrifuged at 12,000 rpm for 20 min at 4°C. The supernatant was filtered through two layers of Whatmann A-1 filter paper. The buffered filtrate acted as enzyme extract.

Assay: Ascorbate peroxidase activity was measured by the method of Nakano and Asada (1987). 3 ml reaction mixture contained 2.5 ml sodium phosphate buffer (pH 7.4, 50 mM) containing 0.3 mM ascorbate and 0.06 mM EDTA, 0.3 ml enzyme extract and 0.2 ml 2 mM \textit{H}_2\textit{O}_2. The decrease in absorbance at 290 nm
(extinction coefficient 2.8 mM⁻¹cm⁻¹) was recorded at 25°C for 1 min. The activity was calculated as µM ascorbic acid oxidized / min / mg protein.

2.11.3: Superoxide Dismutase [EC 1.15.1.1]

Extraction: 100 mg of excised leaf tissues were homogenized in chilled 5ml of Tris HCl (50 mM, pH 7.8) containing 1% PVP and 1 mM EDTA. The homogenate was centrifuged at 14,000 rpm for 15 min. The resulting supernatant was used as enzyme extract.

Assay: The reaction mixture contained 0.24 mM riboflavin, 2.1 mM methionine, 1% triton X 100, 1.72 mM NBT in 50 mM sodium phosphate buffer (pH 7.8) and 200 µl of enzyme extract in a final volume of 3 ml. SOD activity was assayed according to the method of Beauchamp and Fridovich (1971) by measuring ability of the enzyme extract to inhibit the photochemical reduction of NBT. Glass test tubes containing the reaction mixture were immersed in a thermostat bath at 25 ºC and illuminated with fluorescent lamp (Phillips-80 W) for 15 min. Non illuminated identical tubes served as blanks. After illumination for 15 min, absorbance was measured at 560 nm. One unit of SOD was defined as the enzyme activity (per mg protein), which inhibited the photo reduction of NBT to blue formazan, by 50%.

2.12. Antioxidant Levels:

2.12.1. Estimation of L-ascorbic acid:

Ascorbate (reduced form of ascorbate) and dehydroascorbate (oxidized form of ascorbate) were measured based on the reduction of ferric to ferrous ions with ascorbic acid in acid solution followed by the formation of a red-chelate between ferrous ion and 2,2'-bipyridyl (Arakawa et al., 1981 with some modifications). 0.1 g of tissue samples were powdered in Liq.N₂ and homogenized in 2 ml of ice-cold 5% TCA containing 4 % (w/v) PVP-40. The homogenate was filtered through 4 layers of mira cloth and centrifuged at 16,000 x g for 15 min at 4 ºC. The supernatant was used for the AA and total AA (DHA+AA) assay. The reaction mixture for AA assay contained 20 % ethanol, 4% TCA, 0.04 % o-phosphoric
acid ethanol, 0.1% 2, 2'-bipyridyl-ethanol and 0.003% Ferric chloride-ethanol. The reaction mixture was incubated at 30°C for 90 min for the Fe²⁺-bipyridyl complex to develop and the absorbance at 534 nm was recorded. Total AA was determined through a reduction of DHA to AA by dithiothreitol (DTT)-ethanol, after which, 0.24% N-ethylmaleimide (NEM)-ethanol was added in addition to the reaction mixture used for estimating AA and the absorbance of the colour developed was recorded at 534 nm. DHA was measured from the difference of total AA and reduced AA values. The amount of ascorbate present was calculated with reference to a standard curve (Fig-2.5).

![Figure 2.5](image-url) Calibration curve for L-ascorbic acid

**2.12.2 Lipid Peroxidation (MDA):**

Lipid peroxidation was estimated by measuring malondialdehyde (MDA) content spectrophotometrically. MDA determination was carried out according to the method of Heath and Parker (1968). 500 mg leaf was homogenized in 5 ml of 50mM Phosphate buffer pH 7.2 and centrifuged at 10000 x g for 5 min. An aliquot of 0.5 ml supernatant was added to 4 ml TBA-TCA reagent (0.25% Thiobarbituric acid in 10% Trichloroacetic acid) and incubated at 95°C for 30
minutes and again centrifuged at 10000 x g for 15 min. at 4°C. Absorbance was read at 532 nm and value for the non specific absorption was read at 600nm. The amount of Malondialdehyde \( (A_{532}-A_{600}) \) present was calculated from a calibration curve using malondialdehyde as a standard (Fig.2.6).

![Calibration curve for MDA (mg/ml).](image)

**Fig. 2.6 Calibration curve for MDA (mg/ml).**

### 2.14 Statistical analysis

Data are expressed as means ± SEM and were analyzed by the analysis of variance (ANOVA) followed by post hoc Newman-Keuls Multiple Comparison Test (*P < 0.05; **P < 0.01, ***P < 0.001) by using Prism 4 software for windows, Graf Pad Software, Inc, LaJolla, CA, USA.