MATERIALS AND METHODS
### MATERIALS AND METHODS

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MATERIALS AND METHODS

Eight pot and field experiments were conducted on two medicinal plants, viz. *Plantago ovata* Forsk. (isubgol) family Plantaginaceae and *Trigonella foenum-graecum* L. (methi) family Leguminosae during “rabi” (winter) seasons of 1997-99 at the Aligarh Muslim University, Aligarh. The details of the prevailing climatic conditions and soil at Aligarh and of the techniques and procedures employed in each experiment are given below.

3.1 Climatic conditions

Aligarh, an industrial area and a small university town of western Uttar Pradesh (UP), is located 130 km east of Delhi at 27°53' N latitude, 78°4' E longitude and is 187.45 m above the sea level. It has precise climate-wise three seasons, i.e. winter, summer and rainy but generally main crop seasons, viz. “rabi” (winter) and “kharif” (summer) have dominated for centuries. The rabi season extends from the middle of October to the end of March, while the kharif season, from the middle of June to the middle of October. The average recorded day temperature of December and January, the coldest months, is 15°C and 13°C respectively with extreme minimum record of 2.0°C and 0.5°C respectively. May and June are the hottest and driest months, during which the highest temperature has been recorded to reach 45°C whereas the average temperature for May and June is 34.5°C and 34°C respectively (Fig. 2). At the end of June, rainy season (monsoon) starts. The total average rainfall of the year is about 600 mm, of which more than 85 per cent occurs during rainy season (June-September) and the rest in the winter season (Fig. 3).

3.2 Soil characteristics

The soil of Aligarh district is typical of western Uttar Pradesh. The district has sandy, sandy loam and claying loam soil.
3.3 Preparation of the soil for pot experiments

Before the start of the pot experiments, the soil was brought from experimental field at the site of the experiments conducted in pots. After removing weeds and other undesirable particles, the soil was mixed uniformly with the well-rotted farmyard manure in the ratio of 3:1.

3.4 Field preparation

Before the start of each experiment, the field was thoroughly ploughed two times to remove weeds and also to ensure maximum aeration of the soil. After ploughing, the recommended amount of well-rotted farmyard manure, viz. 7.5 t/ha for Plantago ovata Forsk. (Gupta, 1997) and 35 t/ha for Trigonella foenum-graecum L. (Choudhury, 1997) was added uniformly to the field. Required number of beds of 10 square meter (4 m x 2.5 m) was prepared according to the design of the experiment. Before sowing of seeds, a light irrigation was given to maintain the moisture content for the proper germination of seeds.

3.5 Physio-chemical characteristics of soil

Before filling in the pots for each experiment, a soil sample was collected. For the field experiments, small soil samples were collected randomly at different places at the depth of about 10-15 cm. These were mixed together to form a composite sample. Both pot and field soil samples were analysed in the laboratory of the Department of Botany, Aligarh Muslim University, Aligarh. The physio-chemical properties of the soil samples for each experiment are given in Table 1.

3.6 Filling of pots

Each pot (25 cm diameter × 25 cm height) was filled in with 5 kg soil prepared for the pot experiment. The filled pots were arranged according to the design of the experiment and labelled with treatments. Before sowing of seeds, the soil of the pot was irrigated to maintain moisture content for proper germination of seeds.
3.7 Cultural practices

In pot experiment, required amount of nutrients was added at the time of sowing seeds (5 in number) in pot by dipping method. After 2-3 days of germination, one plant was maintained in each pot. The pots were watered as and when required.

In field experiments, the nutrients were applied by broadcasting method at the time of sowing. The seeds were sown in each bed by the hand plough in furrows. Seed rate at 7.5 kg/ha was kept for Plantago ovata Forsk. and 30 kg/ha for Trigonella foenum-graecum L. Rows were separated by a distance of 22.5 cm while the plants, 15 cm.

For Plantago ovata Forsk., nitrogen and phosphorus were given in the form of laboratory grade urea and diammonium phosphate (DAP) in pot experiments and as commercial grade fertilizers field experiments. The amount of nitrogen in DAP was taken into consideration while calculating the required quantity of nitrogen in urea. However, being a leguminous crop, Trigonella foenum-graecum L. was not supplied with nitrogen. Only phosphorus was applied as laboratory grade sodium dihydrogen orthophosphate (NaH₂PO₄.2H₂O) in pot experiments and commercial monocalcium single superphosphate in field experiments. As sufficient quantity of potassium was available in the soil (Table 1), this nutrient was not applied in any experiments.

There were five irrigations for Plantago ovata Forsk. and six irrigations for Trigonella foenum-graecum L. Weeding was undertaken twice in each crop.

When spray of gibberellic acid was included in the scheme of treatments of both pot and field experiments, plants were sprayed with gibberellic acid solution as per treatment on leaves at 50 days stage after sowing (foliage stage). In all experiments, each treatment was replicated thrice. However, in pot experiments, two pots constituted one replicate.

3.8 Experiments 1 and 2

Experiment 1 (pot) and Experiment 2 (field) were conducted simultaneously on Plantago ovata Forsk. during “rabi” (winter) season of
1997-98. The aim of these experiments was to investigate the optimum basal combined dose of nitrogen and phosphorus on the basis of growth, physiological and yield parameters under the agro-climatic conditions of Aligarh. In both experiments, seven combinations of nitrogen and phosphorus were applied. The combinations included: \( N_0P_0 \) (0 kg N + 0 kg P/ha), \( N_{10}P_5 \), \( N_{20}P_{10} \), \( N_{30}P_{15} \), \( N_{40}P_{20} \), \( N_{50}P_{25} \) and \( N_{60}P_{30} \) (Table 2). The design of these experiments was simple randomized. The sowing was performed on 24 October, 1997 and crop was harvested on 15 March, 1998 in both experiments.

3.9 Experiments 3 and 4

Experiment 3 (pot) and Experiment 4 (field) were conducted on *Trigonella foenum-graecum* L. concurrently with the first two experiments. The aim of these experiments was to determine the phosphorus requirement only under the agro-climatic conditions of Aligarh. The requirement of nitrogen is fulfilled by the crop itself by virtue of biological nitrogen fixation. In both experiments, six basal treatments of phosphorus (0, 10, 20, 30, 40 and 50 kg P/ha) were applied (Table 3). The design of these experiments was simple randomized. The sowing was performed on 27 October, 1997 and the crop was harvested on 10 March, 1998 in both experiments.

3.10 Experiments 5 and 6

Experiment 5 (pot) and Experiment 6 (field) were performed together on *Plantago ovata* Forsk. during “rabi” (winter) season of 1998-99. The aim of these experiments was to gain information about the interaction effect of mineral nutrients (nitrogen and phosphorus) and foliar spray of gibberellic acid on the performance of this crop under local conditions in a factorial randomized design. Four selected combination of nitrogen and phosphorus \((N_0P_0, N_{50}P_{25}, N_{60}P_{30}, N_{70}P_{35})\) on the basis of data of Experiments 1 and 2 formed one variant. Four foliar spray treatments of gibberellic acid including water-sprayed control, i.e. 0, 100, 200 and 400 ppm gibberellic acid, comprised
the other variant (Table 4). The crop was sown on 24 October, 1998 and was harvested on 15 March, 1999 in both experiments.

3.11 Experiments 7 and 8

Experiment 7 (pot) and Experiment 8 (field) were conducted on *Trigonella foenum-graecum* L. simultaneously with Experiments 5 and 6. The aim of these experiments was to observe the interaction effect of phosphorus and foliar spray of gibberellic acid under Aligarh conditions. The design of the experiment was factorial randomized. Three treatments of phosphorus (0, 10 and 20 kg P/ha) selected on the basis of the data of Experiments 3 and 4 constituted one factor and four foliar spray treatments of gibberellic acid, i.e. 0, 100, 200 and 400 ppm gibberellic acid, the other (Table 5). The crop was sown on 27 October, 1998 and was harvested on 10 March, 1999 in both experiments.

3.12 Sampling techniques

At the time of sampling, two plants for each replicate were uprooted carefully by the technique employing for pots in pot experiments, while four plants were randomly uprooted from each bed in field experiments. Growth and physiological parameters were studied at 60, 90 and 120 days after sowing (DAS). Yield characteristics were studied at harvest. The details of parameters are given below.

3.12.1 Growth parameters

The following growth characteristics per plant basis were studied:

3.12.1.1 *Plantago ovata* Forsk.

(i) Root length
(ii) Secondary root number
(iii) Root fresh weight
(iv) Root dry weight
(v) Plant height
(vi) Culm number
(vii) Leaf number
(viii) Leaf area
(ix) Plant fresh weight
(x) Plant dry weight

3.12.1.2 *Trigonella foenum-graecum* L.

The following growth characteristics per plant basis were studied:

(i) Root length
(ii) Secondary root number
(iii) Nodule number
(iv) Root fresh weight
(v) Root dry weight
(vi) Plant height
(vii) Branch number
(viii) Leaf number
(ix) Leaf area
(x) Plant fresh weight
(xi) Plant dry weight

3.12.2 Physiological parameters

The following parameters were selected for both crops:

(i) Nitrogen, phosphorus and potassium content in plants
(ii) Transpiration rate
(iii) Stomatal conductance
(iv) Net photosynthetic rate

3.12.3 Yield parameters

The yield performance of the experimental crops was assessed by the following characteristics:

3.12.3.1 *Plantago ovata* Forsk.

(i) Spike number per plant
(ii) Spike length
3.12.3.2 *Trigonella foenum-graecum* L.

(i) Pod number per plant  
(ii) Pod length  
(iii) Seeds per pod  
(iv) 1,000-seed weight  
(v) Seed yield per plant  
(vi) Seed yield per hectare  
(vii) Biological yield per plant  
(viii) Biological yield per hectare  
(ix) Harvest index (HI)

3.12.4 Determination of nitrogen, phosphorus and potassium content in plants

In all the eight experiments, the sampled plants were analyzed at various growth stages for assessing the nitrogen, phosphorus and potassium status. The sampled plants were dried for 24 hours in an oven at 80°C. These dried samples were finally powdered and passed through a 72-mesh screen. The dried powder was digested according to Lindner (1944).

3.12.4.1 Digestion of sample powder

100 mg oven dried powder was carefully transferred to a digestion tube and 2 ml pure sulphuric acid was added to this digestion tube and was kept in digestion assembly at 80°C for about 2 hours to allow complete reduction of nitrates present in the plant material. Initially, dense white fumes were given
off and then the contents turned black. After two hours, the digestion tube was cooled followed by dropwise addition of 0.5 ml of 30% H₂O₂(w/v). The digestion tube was heated further for about 30 minutes till the colour of the contents changes from black to light yellow. The digestion tube was cooled for 10 minutes and additional amount of 2-3 drops of hydrogen peroxide was added followed by gentle heating for about 15 minutes to get clean and colourless solution. At this stage, care was taken in the addition of hydrogen peroxide because its excess might oxidise ammonia in the absence of organic matter. The sulphuric acid peroxide digested material was diluted with double distilled water and transferred with three washing to a 100 ml volumetric flask and finally the volume was made up to the mark (100 ml) with double distilled water. Appropriate aliquot was used to estimate nitrogen, phosphorus and potassium. The details of methods employed for the analysis of these elements are given below.

3.12.4.2 Estimation of nitrogen

The nitrogen was estimated by the method of Lindner (1944).

A 10 ml aliquot of the sulphuric acid peroxide digested material was taken in a 50 ml volumetric flask and the excess of the acid was neutralized by the addition of 2 ml of 2.5N sodium hydroxide (Appendix). 1 ml of 10% sodium silicate (Appendix) was added to prevent turbidity and finally, the volume was made up with double distilled water.

3.12.4.2.1 Development of colour

A 5 ml aliquot of this solution was taken in a 10 ml graduated test tube and 0.5 ml of Nessler's reagent (Appendix) was added dropwise mixing it thoroughly after each addition.

After adding distilled water to make up the volume of contents were allowed to stand for about five minutes for maximum colour development.

The solution was transferred to a colorimetric tube and transmittance was read on spectrophotometer (Spectronic 20D, Milton Roy, USA) at 525 nm.
A blank was run with each set. A standard calibration curve was obtained by using known dilution of ammonium sulphate solution.

3.12.4.2.2 Procedure for standard calibration curve

50 mg ammonium sulphate was dissolved in one litre double distilled water. From this stock solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml was pipetted to ten different test tubes. The solution in each test tube was diluted to 5 ml with double distilled water. In each test tube, 0.5 ml Nessler's reagent was added. After 5 minutes, the solution was transferred to a colorimetric tube and transmittance was read on spectrophotometer (Spectronic 20D, Milton Roy, USA) at 525 nm. A blank was run with each set.

A curve was plotted with nitrogen concentration as x-axis and optical density as y-axis. Optical density (OD) was determined by the following formula:

\[ OD = 2 - \log T \]

Where, \( T \) is per cent transmittance.

3.12.4.3 Estimation of phosphorus

Phosphorus was estimated according to method of Fiske and Subba Row (1925).

A 5 ml aliquot of the sulphuric acid peroxide digested material was taken in a 10 ml graduated test tube and 1 ml molybdc acid (Appendix) was carefully added followed by the addition of 0.4 ml 1-amino-2-naphthol-4-sulphonic acid (Appendix). The colour of the solution was turned blue. Double distilled water was added to make up the volume to 10 ml and the solution was allowed to stand for five minutes after mixing thoroughly. It was then transferred to a colorimetric tube and the optical density was read at 620 nm on spectrophotometer (Spectronic 20D, Milton Roy, USA). A blank was run simultaneously for each determination.
3.12.4.3.1 Procedure for standard calibration curve

0.351 g potassium dihydrogen orthophosphate (KH₂PO₄) was dissolved in double distilled water and 10 ml 10N sulphuric acid was added and the volume was maintained one litre with double distilled water.

From this stock solution 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and 1.0 ml concentrations were taken from the above solution in 10 different test tubes and 1 ml molybdic acid and 0.4 ml 1-amino-2-naphthol-4-sulphonic acid was added in each test tube and the volume was made upto the mark. The reading was directly read at 620 nm on spectrophotometer (Spectronic 20D, Milton Roy, USA). A blank was also run with each set of determination.

3.12.4.4 Estimation of potassium

Potassium content was estimated by flame photometrically.

A 10 ml aliquot of peroxide digested material was taken and it was read on flame photometer. A blank consisting of double distilled water only was run side by side. The readings were compared with the calibration curve plotted for different dilutions of a standard potassium chloride solution.

3.12.4.4.1 Procedure for standard calibration curve

1.907 g potassium chloride was dissolved in 100 ml double distilled water, of which 1 ml solution was diluted to one litre. The resulting solution would be of 10 ppm K. From 10 ppm K solution, 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 ml solution was transferred to 10 ml vials separately. The solution in each vial was diluted to 10 ml with double distilled water. The diluted solution of each vial was run separately. A blank was run with each set of determination. Standard curve was prepared for different dilutions of potassium chloride solution versus readings on the scale of the galvanometer. With the help of the standard curve, the amount of potassium content in the sample was determined.
3.12.4.5 Determination of transpiration rate, stomatal conductance and net photosynthetic rate

Transpiration rate, stomatal conductance and net photosynthetic rate were measured on cloudless clear days at 11:00 A.M. with Ca = 0.33 m mol CO₂ mol⁻¹ (330 µL/L) on fully expanded leaf in both plants (*Plantago ovata* Forsk. and *Trigonella foenum-graecum* L.) in all experiments with the help of IRGA (Infra Red Gas Analyzer, LI-COR 6200) portable photosynthesis system (Nebraska, USA). Before taking the measurement, the IRGA (LI-COR 6200) was calibrated and the zero was adjusted approximately every 30 minutes during the measurement period. During this measurement, leaf was enclosed in a one litre gas exchange chamber for 60 seconds. All above parameters were measured three times in each treatment in all experiments at following measurement:

(i) Barometric pressure = 979.9 mb
(ii) One sided boundary layer conductance = 1.350 mol m⁻² s⁻¹
(iii) Volume of entire system [Vt(CC)] = 1149 cm²
(iv) The IRGA volume, including hoses to the chamber [Vg(CC)] = 154 cm³
(v) An estimate of the ratio of stomatal conductance of one side of the leaf to the other (STMART) = 1.0000
(vi) The maximum flow rate that could be achieved through the desicant = 999.9 (µmol)
(vii) Water absorption factor = 1.500

3.12.4.6 Determination of harvest index (HI)

Harvest index was calculated by the following formula:

$$HI = \frac{\text{Seed yield}}{\text{Total biomass}} \times 100$$
3.12.4.7 Determination of swelling factor

The swelling factor (mucilage content) was measured according to the method given in pharmacopoeia of India (Anonymous, 1955) and summarized below:

1 g seeds of *Plantago ovata* Forsk. were agitated gently and occasionally for 24 hours in a 25 ml stoppered measuring cylinder filled upto 20 ml mark with double distilled water. Therefore, they were allowed to stand undisturbed for one hour so as to occupy a volume of not less than 10 ml. The swelling property of the seeds was measured by reading the actual level of swelling mass in the measuring cylinder, i.e. the final volume occupied by it as a result of adsorption of water.

3.13 Statistical analysis

The statistical analysis of the data was done according to Gomez and Gomez (1984) to find out the significance of the effect of various treatments as per design of each experiment. F' test was applied to determine the significance of the data at 5% level of probability. Critical difference (CD) among the values was also calculated to determine the statistical significance of the data. The model of analysis of variance (ANOVA) for each of the experimental design was given in Tables 2-5.

The correlations of various parameters were determined by calculating correlation coefficient (r). The values for correlation coefficient were calculated using the formula:

\[ r = \frac{\sum (x - \overline{x}) (y - \overline{y})}{\sqrt{\sum (x - \overline{x})^2 \cdot \sum (y - \overline{y})^2}} \]

where, \( x \) = Independent parameter

\( (\overline{x} = \text{mean}) \)

\( y \) = Dependent parameter

\( (\overline{y} = \text{mean}) \)
To test the significance of the r value the following formula was applied:

\[ \text{Test of significance (t')} = r \sqrt{\frac{n - 2}{1 - r^2}} \]

Where, \( n \) = number of total observations

= number of total treatments × number of replicates

If t' value was found greater than that of predicted t' value, obtained from the table at 1% and 5% levels of probability, the value for correlation coefficient was declared to be significant in that order.
CHAPTER 3 – MATERIALS AND METHODS

The parameters selected were those that may help in the proliferation and increase in the above ground plant biomass. The parameters like fresh weight, plant height, culm number, spike and pod length may ascribe number of leaves, branch number and seed output due to nutrients supply and its interaction with GA3. Total nutrient uptake and biological yield and their relationship has been worked out and presented in Tables 120-127 for each experiment. Although plasma membrane transporters activity was not worked out but the increase in above-ground plant growth with either nutrient or GA3 treatments or their interaction accumulated more nutrients through signalling roots. Unit leaf rate was not included in the thesis considering the volume of the thesis and also that this is a secondary data. The primary data on leaf surface area and photosynthetic rate were included. However, in this supplement data on ‘crop growth rate’, ‘relative growth rate’ and ‘net assimilation rate’ are included (Tables 144-159). Since the crops taken for study are of medicinal importance, its fresh mass and seeds both are important. An increase in above-ground plant proliferation is useful and is expected to have increased seed yield through translocation efficiency to sink.

The other details required in revision are as follows:

- GA3 was dissolved in sufficient amount of ethyl alcohol and required concentrations were prepared in water.
- The potted plants were grown under natural day/night conditions (PAR > 1000 μ moles/m²/s; temperature day 22°C, night 12°C; relative humidity 67%).
- Field-grown plants were uprooted carefully to avoid root damage and root length was measured with meter scale.
- Leaf numbers included ‘functional leaves’.
- Leaf area was measured gravimetrically.
Net photosynthetic rate was measured on youngest fully expanded leaves on the axis of plant at PAR > 1000 μ moles/m^2/s between 11.00-12.00 hours. The PAR was at light saturation for photosynthesis. Intercellular CO₂ concentration was also determined and given with this supplement (Tables 160-165).