CHAPTER – 3

MATERIALS AND METHODS
STUDY SITE DESCRIPTION:

The study site was around Udham Singh Nagar selected for study of AM fungal association with spice crops. The material for study was collected at Udham Singh Nagar and of Uttrakhand state at 29°58'N latitude and 76°5' longitude and at about 250m above msl. This area is tropical in its climate, receiving annual rainfall between 500-1500mm commonly during monsoon period (June-Sept). Seasons which are found in this area, summer (March-May) which is hot and dry, rainy (June-Sept) warm and wet and winter (Oct-Feb) cool and dry. The mean maximum temperature varies from 17°C (Jan) to 38±2°C (May) and mean minimum temperature varies from 4.1°C (Jan) - 26°C (May).

Udham Singh Nagar district falls in the Tarai region of Kumaon Division. Soils of district U. S. Nagar is alluvial in nature. Drainage of soil is imperfect and water logging is great threats to the field crops. Soil is calcareous, productive and suitable for extensive cultivation of high yielding variety of crops.

_Capsicum annuum_ is an annual plant. _Capsicum annuum_ belongs to the genus Capsicum under Solanaceae family. The flowers are an off-white (sometimes purplish) colour while the stem is densely branched and up to 60 centimetres (24 in) tall. The fruit is a berry which may be green, yellow or red when ripe. _C. annuum_ requires a warm and humid climate for its best growth and dry weather during the maturation of fruits. Heavy rainfall leads to poor fruit set and in association with high humidity leads to rotting of fruits. A temperature ranging from 20-25°C is ideal for _C. annuum_. In _C. annuum_ fruit development was found to be adversely affected at 37°C temperature or more. High temperature associated with low relative humidity at flowering increases the transpiration resulting in shedding of buds, flowers and small fruits. _C. annuum_ can be grown in a range of soils, but black soils which retain moisture for long periods are suitable for rainfed crop whereas well drained soils, deltaic soils and sandy loams are good under irrigated condition.
VAM FUNGI-

*Glomus* is the genus in the family Glomeraceae, in the division Glomeromycota. All Glomalean fungi produced arbuscules to form mycorrhiza. Arbuscules are intracellular, haustoria like structures formed by repeated branching. At this interface phosphates and fixed carbon molecules are exchanged. Spore colour varies from hyaline to black and texture from smooth to highly ornament. Spores usually develop thick walls with more than one layer and function as storage structures, resting stages and propagules. Spore size varies from 10µm to more than 1000µm.

*Trichoderma* is the genus in the family Hypocreaceae, in the division Ascomycota. *Trichoderma* is present in all soils, where they are the most prevalent culturable fungi. Conidiophores are highly branched and thus difficult to define measure, loosely or compactly tufted, often formed in distinct concentric rings or borne along the scant aerial hyphae. Main branches of the conidiophores produce lateral side branches that may be paired or not, the longest branches distant from the tip and often phialides arising directly from the main axis near the tip. Phialides are typically enlarged in the middle but may be cylindrical or nearly subglobose. Phialides ay be held in whorls, at an angle of 90° with respect to other members of the whorl, or they may be variously penicillate. Phialides may be densely clustered on wide main axis or they may be solitary. Conidia typically appear dry but in some species they may be held in drops of clear green or yellow liquid. Conidia are typically smooth but tuberculate to finely warded conidia are known in a few species.

*Acaulospora laevis* is a species of fungi in the family Acaulosporaceae, in the phylum Glomeromycota. It forms arbuscular mycorrhiza and vesicles in roots. Spores are formed laterally on the neck of a small thin walled saccule called a mother spore or vesicle or sporogenous saccule. Spores are globose to ellipsoid. The surface of the spore wall may be ornamented with pits, projections, spines or reticulations. Spores are generally honey colored, sessile in nature, sometimes spores show expressions of hyphal attachment known as eccetrix, walls thick at eccetrix point, wall layer smooth with homogenous matrix, 200-400 µm in diameter, ellipsoid to egg-shaped generally.
**SAMPLING -**

The collection of roots and soil samples of some spice plants was done. The soil samples of three species were taken and fine roots were collected in polythene bags from each plant. About 200-250 gm of soil samples from rhizosphere of each plant at a depth of 15-30cm was collected in polythene bags. These samples were mixed to form a composite sample and then brought to laboratory and proceed for isolation of AM fungal spores. Root and soil sample from the rhizosphere of each plant upto depth of 15-30cm were collected at flowering stage in polythene bags. These samples were brought to the laboratory for mycorrhizal quantification and root colonization and stored at 5-10°C.

**ISOLATION OF AM FUNGI -**

'Wet-Sieving and Decanting technique' (Gerdemann and Nicolson, 1963) was used for isolation of AM fungi spores. For this, sieves of different sizes i.e. 150µm, 120µm, 90µm, 63µm and 45µm were used. Soil sample (50gm) was throughly mixed in water in a beaker of 500ml using magnetic stirrer and allowed to settle overnight. Place the sieves in the following order 150µm, 120µm, 90µm, 63µm and 45µm from top to bottom. The water of the beaker was decanted on a series of sieves, on which spores were trapped and then they were washed with running tap water. The trapped spores were transferred to Whatman filter paper No. 1 by repeated washing with water. Spores were picked by hypodermic needle under stereo binocular microscope. The spores were mounted on polyvinyl lactic acid alcohol (PVLA) for further observations.
**MYCORRHIZAL QUANTIFICATION** -

Quantitative estimation of AM fungi spores was done by modified Grid line intersect method given by Adholeya and Gaur in 1994. In this method, the filter paper was divided into many small compartments by a ball point pen and each compartment was numbered. The total number of spores counted under stereobinocular microscope by using counter.

**IDENTIFICATION OF AM FUNGI** -

The main structure of AM fungi is spores used for identification. Following morphological criteria viz., colour size, shape, wall structure, bulbous suspensor, the number and arrangement of spores in the sporocarps were used for AM fungi identification. These AM fungi spores were identified using identification manual of Walker (1983), Sheneck and Perez (1990), Morton and Benny (1990) and Mukerji (1996).

**MYCORRHIZAL ROOT COLONIZATION** -

'Rapid clearing and staining technique' of Phillip and Hayman (1970) was used for studying mycorrhizal root colonization. For this roots were washed under tap water to remove adherent soil particles. Then they were cut into 1cm long bits and placed in 10% KOH at room temperature for 24 hours. After this, decant KOH and root pieces were washed with water to remove brown colour. Then, roots were acidified with 1% HCl for 3-5 minutes. Acid was decanted and root pieces were stained with 0.5% Trypan blue for 24hrs. After that the trypan blue was decanted and these root pieces were destained with Lactophenol to remove excess of stain. Mounting of roots were done in Lactic acid or Lactic acid - Glycereol (1:1) solution.
The total percent of root colonization was determined by the Root slide technique, as described by Giovannetti and Mosse (1980). The following formula was used to calculate the percent root colonization.

\[
\text{Percent mycorrhizal root colonization} = \frac{\text{No. of root segment infected}}{\text{Total No. of Root segments studied}} \times 100
\]

**DIFFERENT TREATMENTS OF AM FUNGI AND TRICHODERMA HARZIANUM -**

In the present investigation, *Glomus mosseae*, *Acaulospora laevis* and *Trichoderma harzianum* single and in double combination with each other were screened to see their effect on *Capsicum annuum*. The different combination used were given as follows -

1. Control
2. *Glomus mosseae*
3. *Acaulospora laevis*
4. *Trichoderma harzianum*
5. *G. mosseae* + *T. harzianum*
6. *A. laevis* + *T. harzianum*
7. *G. mosseae* + *A. laevis*
INOCULUM PRODUCTION -

MASS PRODUCTION OF *GLOMUS MOSSEEAE* AND *ACAULOSPORA LAEVIS* -

**STARTER INOCULUM PRODUCTION FOR AM FUNGI**

Each selected AM fungi were raised by 'funnel technique' (Menge and Timmer, 1982) using sorghum as host for starter inoculum. In the funnel technique, spores were isolated by 'wet sieving and decanting technique' on whatman filter paper. Then under stereobinocular microscope, spores were picked. After that these spores were surface disinfected using 2% (w/v) chloramines – T for 15 min. and rinsed thoroughly in sterilized water. The spores viability tested by Thionine stain, which turns viable spores into dark blue colour. Ten healthy and viable spores were selected and mass produced in funnels using sorghum as host. The roots were studied for AM root colonization after 30 days and for spore quantification 10gm of soil samples were studied.

**SELECTION OF HOST AND SUBSTRATE** -

For maximum root colonization of *G. mosseeae* and *A. laevis*, *Sorghum vulgare* was used as host and a mixture of soil and sand (3:1) was used as substrate. Substrate was sterilized in autoclave at 15 IB pressure for 30 minutes.

**MASS PRODUCTION OF TRICHODERMA HARZIANUM** -
*T. harzianum* colonies were first grown on PDA (Potato Dextrose Agar) medium for 10-12 days and incubated at 25-30°C in BOD for maximum growth and sporulation. Then the inoculum containing medium were cut into 8mm small round discs with the help of sterilized cork borer and 8-10 fungal discs were poured into the flask containing the wheat bran-saw dust-corn flour medium (substrate) for mass production of *Trichoderma harzianum*.

**MASS INOCULUM OF *Fusarium oxysporum***

The inoculum of *F. oxysporum* was first grown on PDA medium at 25-30°C in B.O.D. incubator for 10-15 days for maximum growth and sporulation. Then the inoculum containing medium was cut into small discs and were put in flasks containing 0.1% liquid Agar medium for mass multiplication of *F. oxysporum*. Ten percent of the inoculum was used for each treatment.

**SOIL STERILIZATION AND FILLING OF POTS***

For experiment the pots of 35×25 cm were selected and soil was collected from top 10 cm of soil. The soil was dried in air and sieved through a 2mm sieve to remove stone and plant debris and after that it was sterilized in autoclave for 1 hour at 15 lbs pressure and 121°C temperature and then cool it at room temperature. After following this pots were filled with 1000 gm of soil.

**TEST PLANTS***

We obtained Monsanto Holdings Private Limited branded seeds from local seed market of Kashipur as mostly obtained by the farmers of this area. Thereafter in the month of May, we sowed these seeds in the nursery. After 3 weeks *Capsicum* seedlings were procured from this nursery. For experiment 25 pots were selected. In each pot five seedlings were planted, single and in double combination and plants were placed in the polyhouse.
Plants were watered regularly as required. Hoagland's nutrient solution was given to plants (100ml/pot) after regular interval of 15 days.

**INOCULATION -**

i) Single inoculation – For single inoculation, 150gm of soil of each inoculant (\textit{G. mosseae, A. laevis and T. harzianum}) were added in each pot except control. It was again covered with 300gm of sterilized soil.

ii) Double inoculation – For double inoculation, 75gm + 75gm of soil of each inoculum was added in each pot. The different combinations were used as following.

(a) \textit{G. mosseae} + \textit{T. harzianum}

(b) \textit{A. laevis} + \textit{T. harzianum}

(c) \textit{G. mosseae} + \textit{A. laevis}

It was again covered with 300gm of sterilized soil.

**SELECTED PARAMETERS FOR GROWTH RESPONSE -**

During experiment, growth parameters were selected for recording the growth response in the inoculated and control plants after 45 and 120 days from transplanting.

1. Shoot length (cm) of the longest branch.
2. Root length (cm) of primary root.
3. Fresh shoot weight (gm)
4. Dry shoot weight (gm)
5. Fresh root weight (gm)
6. Dry root weight (gm)
7. Mycorrhizal root colonization (%)
8. Mycorrhizal spore count per 10 gm of soil
9. Phosphorus content in root and shoot (%)

ROOT LENGTH -

Roots were harvested after 45 and 120 days from each pot. Roots were washed under tap water to remove adherent soil particles. Then root length was measured with the measurement tape.

PHOSPHORUS ESTIMATION OF PLANT SHOOT AND ROOT -

Total phosphorus content was determined by 'Phospho Vanadomolybdate yellow colour method' given by Jackson (1973)

PROCEDURE -

i) DIGESTION OF PLANT SAMPLES -

Plants were harvested after 45 and 120 days from each pot. Both plant root and shoot were washed properly with tap water then washed again with distill water followed by blotted dry and kept in an oven at 70°C until completely dried. After complete drying these was ground separately into fine powder and only 100mg of it was taken into 100ml conical flasks. Wet digestion was done by addition of 10ml di-acid mixture of nitric acid and perchloric acid (3:1) into each flask containing samples. The flasks were placed on a hot plate for 1-2 hrs (for complete digestion)
and care was taken to avoid drying of the samples. After cooling, the digested samples content were transferred to 100ml. volumetric flasks and volume was raised upto the mark of 100ml with the double distilled water. The solution was filtered through Whatman filter paper no.1 to remove hydrated silica and filterate was used for the further analysis of phosphorus.

(ii) PHOSPHORUS AMOUNT -

Total phosphorus amount after digestion was estimated as follows- Ammonium-molybdate-vanadate reagent was prepared by dissolving 22.5gm of ammonium molybdate \((\text{NH}_4)_6\text{Mo}_7\text{O}_{24}\) in 400ml of distilled water and 1.2gm of ammonium-m-vanadate in 300ml of hot distilled water and after that mixed the above both solutions and left it to cool down to room temp.followed by adding 250ml of conc. nitric acid and volume was raised upto 1000ml in volumetric flask. Phosphorus standard solution was prepared by dissolving 0.11 gm of \(\text{KH}_2\text{PO}_4\) is distilled water and diluting it to 1 litre. This standard solution contains 25ppm of phosphorus. One ml of aliquot of acid digest was transferred in a test tube and added 1 ml of colouring agent to it. After mixing it was diluted to 10ml with distilled water and shake it for mixing. After 30 minutes, the colour intensity was read at a wavelength of 420nm in an UV-visible recording spectrophotometer. Phosphorus was calculated from the standard curve prepared similarly in the range of 2.5-10ppm of phosphorus.

DIFFERENT TREATMENTS OF AM AND TRICHODERMA HARZIANUM AGAINST FUSARIUM OXYSPORUM-

In the present investigation, \textit{T. harzianum} was screened alone and in combination with \textit{Glomus mosseae, Acaulospora laevis} to see the biocontrol of wilt in \textit{Capsicum annuum} caused by \textit{Fusarium oxysporum}.
The different combination were –

1. *Fusarium oxysporum* (control)

2. *Glomus mosseae* + *F. oxysporum*

3. *Acaulospora laevis* + *F. oxysporum*

4. *Trichoderma harzianum* + *F. oxysporum*

5. *G. mosseae* + *A. laevis* + *T. harzianum* + *F. oxysporum*

**INOCULATION -**

i) Control - For control, 150 ml. of liquid both containing *Fusarium oxysporum* inoculum was added in each pot and mix it with the soil. It was again covered with 250 gm of sterilized soil.

ii) Double Inoculation - For double inoculation, 75 ml + 75 gm of each inoculum was added. It was again covered with 250 gm of sterilized soil. The different combinations of inoculum were-

A- *G. mosseae* + *F. oxysporum*

B- *A. laevis* + *F. oxysporum*

C- *T. harzianum* + *F. oxysporum*

iii) Quadruple Inoculation - For quadruple inoculation, inoculum approximately 40g+40g+40g+40ml of each inoculum was added. It was again covered with 250 gm of sterilized soil.

The combination of inoculum was -

*G. mosseae* + *A. laevis* +*T. harzianum* + *F. oxysporum*
SELECTED PARAMETERS FOR GROWTH RESPONSE -

Following parameters were selected for recording the biocontrol of experimental plant after 80 days.

1. Shoot height (cm)
2. Root length (cm)
3. Fresh and dry shoot biomass (gm)
4. Fresh and dry root biomass (gm)
5. Mycorrhizal root colonization (%) and spore number.