Material and Methods
A) Studies on seed-borne fungi:

1) Collection of seed samples

The method described by Neergaard (1973) has been adopted for the collection of seed samples. Accordingly, three random samples of seeds (half kg of each variety) were collected from the fields, store houses and market places. A composite sample of this was made by mixing the individual samples together, preserved in cloth bags at room temperature during the period of studies.

2) Detection of seed mycoflora

The procedure for blotter test and agar plate methods as recommended by International Seed Testing Association (ISTA) 1966, De Tempe (1970), Neergaard (1973) and Agrawal (1976) were adopted in the present studies, unless otherwise stated.

a) Standard Blotter method:

A pair of white blotter papers of 8.5cm diameter was jointly soaked in sterile distilled water, placed in pre-sterilized corning petriplates of 10cm diameter. Ten seeds per petriplate were placed at equal distance on the moist blotters. One hundred seeds were employed in every experiment. The plates were incubated at 25±2°C under diurnal conditions. On seventh day
of incubation the seeds were examined under stereoscopic microscope for the preliminary determination of fungal growth on them. The identification and further confirmation of seed-borne fungi was made by preparing slides of the fungal growth and observing them under compound microscope.

b) Agar plate method:

In this method, pre-sterilized corning glass petriplates of 10cm diameter were poured with 20ml of autoclaved potato dextrose agar (PDA) medium. On cooling the medium, 10 seeds per petriplate were placed at equal distance aseptically. Incubation conditions and other details were same as described for the blotter test method.

In order to isolate only internal seed-borne mycoflora, seeds were pretreated with 0.1 percent solution of mercuric chloride for 2 minutes and subsequently thoroughly washed thrice with sterilized distilled water and placed on agar plates. Seeds without any such pre-treatment were employed for the study of total seed mycoflora.

c) Identification of seed-borne fungi:

The fungi occurring on each and every in the plates were identified preliminary on the basis of sporulation characters like sexual or asexual spores or fruiting structures with the help of stereoscopic microscope. Detailed examination of fungal characters was done under compound microscope and their identification was made with the help of manuals. Pure cultures of these fungi were prepared and maintained on potato dextrose agar (PDA) slants.
3) Composition of media used in isolation:

I) Potato Dextrose Agar (PDA):

200g peeled potatoes were boiled until soft and passed through muslin cloth, 20g of dextrose was added to it and final volume of solution was made upto 1000ml. In this 20g of Agar agar was added, pH of the medium was adjusted to 5.6.

II) Czapek Dox Agar [CZA]:

Sucrose-15g, NaNo₃-2g, KH₂PO₄-0.1g, MgSO₄·7H₂O-0.5g, KCl-0.5g, FeSO₄·7H₂O-0.01g, Agar-15g and distilled water-1000ml.pH 5.6.

B) Studies on protease production:

a) Production:

Production of protease(s) was made by growing the fungi on liquid medium containing glucose 10g, gelatine 10g dipotassium hydrogen phosphate 1.0g, MgSO₄·7H₂O-0.5g and distilled water 1000ml. PH of the medium was adjusted at 5.5. 25ml of the medium was poured in 100ml Erlenmeyer conical flask and autoclaved at 15lbs pressure for 20minutes. The flasks on cooling were inoculated separately with 1ml standard spore or mycelial suspension of test fungi prepared from 7 days old cultures grown on PDA slants. The flasks were incubated for 6 days at 25±1°C with diurnal periodicity of light. On 7th day, the flasks were harvested by filtering the contents through Whatman No.1 filter paper. The filtrates were collected in pre-sterilized bottles and termed as crude enzyme preparations.
b) Assay method:

1) Cup plate method

Determination of protease(s) activity was done with the help of cup plate method, adopted by Hislop et. al., (1982) and Rajamani (1990). A basal medium was prepared by adding 2 percent (W/V) agar and one percent (W/V) gelatine, pH of the medium was adjusted at 5.6 with McIlvaine buffer. Then it was sterilized at 15lbs pressure for 15 minutes. About 15 ml of the medium was poured in pre-sterilized petriplates under aseptic conditions. On solidifications 6 mm diameter cups or cavities were made in the centre of each of the agar plate with a sterilized cork borer (No.4). The cups or cavities were filled carefully with about 0.5ml of culture filtrate (crude enzyme preparation). The plates were incubated at 25±2°C for 24 hours. Then the plates were flooded with 15 percent mercuric chloride (HgCl₂) in 7NHCl. After 10 minutes of standing, a clear transparent zone indicated the hydrolysis of gelatine by extracellular proteolytic enzymes, where as the rest of the region of the petriplates become opaque due to the coagulation of gelatine (protein) by mercuric chloride. Diameter of the clear zone was used as a measure (mm) of protease activity, while non appearance of clear zone considered absence of protease(s) in the culture filtrates.
Composition of media used for protease production:

Various synthetic media were employed for the production of protease(s) in the preliminary experiment. Composition of the media is given below.

I) Glucose nitrate medium (GN)

Glucose, 10g Kno₃, 2.5g, KH₂PO₄ 1.0g, and MgSO₄·7H₂O-0.5g, dissolved in 1000ml distilled water.

II) Glucose gelatin medium:

Glucose 10g, Gelatine 10g, K₂HPO₄ 1.0g and MgSO₄·7H₂O 0.5g, dissolved in 1000ml distilled water.

III) Gelatin broth:

Gelatin 10g, K₂HPO₄ 1.0g and MgSO₄·7H₂O 0.5g, dissolved in 1000ml distilled water.

IV) Glucose Asparagine medium:

Glucose 10g, Asparagine 5g, K₂HPO₄ 1.0g and MgSO₄·7H₂O 0.5g, dissolved in 1000ml distilled water.

V) Glucose peptone medium:

Glucose 20g, Peptone 4.5g, Asparagine 4.5g, K₂HPO₄ 3.4g and MgSO₄·7H₂O 1.9g and NaCl 0.01g, dissolved in 1000ml distilled water.

C) Studies on Phytotoxins:

a) Production of phytotoxins:

The test fungi isolated from pea seeds were grown on Richard’s medium. 25ml of the medium was added in 100ml conical flasks and
autoclaved at 15lbs pressure for 15min. On cooling, flasks were inoculated separately with 1ml of spore suspension of test fungi prepared from 7 days old cultures grown on PDA slants. The flasks were incubated at 25±2°C for 9 days and were harvested by filtering their contents through whatman No.1 filter paper. The filtrates were collected in presterlized bottles and termed as crude toxin preparations. These preparations were tested for their toxicity.

b) Assay method:

The toxicity of culture filtrate was determined by using following methods.

I) Seed germination method:

Surface sterilized hundred seeds of each variety were soaked in crude toxin preparation for 24 hours. They were then placed on moist blotter in petriplates. Seed soaked similarly in freshly prepared uninoculated liquid medium served as control. Percent germination or percent inhibition of germination, root and shoot length of seedlings were measured after 7 days of incubation at room temperature.

II) Wilting of shoots:

Shoots of pea grown in the field were used for the test 5ml culture filtrate was taken in a vial in which shoot was dipped to the level of culture filtrate and incubated for 24 hours at room temperature. Shoots kept in freshly prepared sterile medium served as control. The wilting symptoms caused due to the culture filtrates were recorded by using the following scale.
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0 = No Damage  
1 = Stem dropping  
2 = Leaves Collapse  
3 = Leaves soaked  
4 = Leaves dry brittle

Composition of media used for phytotoxin production:

1. Glucose nitrate medium:

   Glucose 10g, KNO₃ 2.5g, KH₂PO₄ 1.0g, and MgSO₄·7H₂O 0.5g, dissolved in 1000ml distilled water.

2. Richard’s medium:

   Sucrose 50g, KNO₃ 10g, KH₂PO₄ 5.0g, MgSO₄·7H₂O 2.5g and FeCl₃ 0.02g, dissolved in 1000ml distilled water.

3. Czapek’s medium:

   Sucrose 30g, NaNO₃ 2.0g, K₂HPO₄ 1.0g, MgSO₄·7H₂O 0.5g, KCl 0.5g and FeSO₄·7H₂O 0.01g, dissolved in 1000ml distilled water.

Biodeterioration of seeds

For this, freshly harvested mature and apparently healthy seeds were collected from the fields. They were surfaced sterilized with 0.1% mercuric chloride solution and subsequently washed and soaked in sterile distilled water for four hours. Excess water was decanted from the seeds. The seeds were distributed into three flasks (100g per flask) and were inoculated separately with 2ml spore suspension of the test fungi. The flasks were incubated at room temperature for various period ranging from 10, 20, 30 days and were harvested for studying physical and chemical changes in the seeds.
At the time of harvest, seeds were thoroughly washed under running tap water in order to remove complete mycelial growth from their surface. Subsequently, the seeds were dried at 60°C for 48 hours and crushed into fine powder for the estimation of different chemicals. Seeds incubated in a similar manner but without inoculating spore suspensions of fungi served the control.

**Estimation of Crude fat:**

The crude fat in the plant material was estimated by the standard Soxhlet method given in (A.O.A.C., 1970). The fats present in the plant material are extracted in the solvent consisting of chloroform (CHCl₃) and methanol (CH₃OH). This is done in Soxhlet extraction assembly and after complete evaporation of the solvent, the amount of extracted fat is measured.

Weigh 2g dry seed powder and transfer it into a thimble prepared with Whatman filter paper No.1. Plug the mouth of thimble with fat free absorbent cotton. Take clean, dry 250ml receiver flask from the Soxhlet assembly and add the solvent to it just to reach the level of the neck. Introduce the thimble with sample into the Soxhlet. Assemble the apparatus and place it on heating mental with temperature controlling device. Fit water condenser at the top of the Soxhlet. Extract the fat for 8 hours at 60°C. When the extraction is over, remove the thimble from Soxhlet. Assemble the apparatus again and heat to recover most of the solvent from the receiver flask. When the receiver flask contains about 25ml solvent
along with the extracted fat, disconnect the receiver flask. Transfer the solvent in a clean previously weighed beaker with rising 2 to 3 times. Dry it in a hot air oven at 95°C, cool in a dessicator and weigh. Measure the amount of fat, extracted per 2g of the sample and calculate the amount of crude fat as percent of dry matter (DM)

**Estimation of Ash:**

The residue after incineration of sample at 550-600°C is known as ash. For this purpose the sample is subjected to a high temperature upto 600°C and then the ash content is determined. During ignition to such a high temperature all organic compounds decompose and pass off in the form of gases, while the material elements remain in the form of ash. For this the procedure is followed by A.O.A.C. (1970) and Mungikar (1999).

2g of oven dry seed powder was placed in a previously weighed crucible and it was subjected for heating on hot plate till the sample was sufficiently turned black about 30 minutes. Then it was placed in muffle furnace, pre-heated to 600°C for 2 hours with automatic control. Crucible were transferred directly to dessicator, cooled and weighed immediately. Weigh of ash was obtained per 2g of sample and further calculated the ash content as percent of Dry Matter (DM).

**Estimation of crude protein:**

This was done by estimating N content in the samples with the help of microkjeldahl technique (AOAC, 1970). The amount of N content was multiplied by 6.25 factor which gave crude protein content of the samples.
300mg seed powder were taken in Kjeldahl flask along with 250g $K_2$SO$_4$ and 40mg CuSO$_4$ and kept overnight. This was digested till the mixture become white. After complete digestion the flasks were allow to cool. The digest was processed for distillation with the help of markham’s distillation set.

Digest was diluted to 50ml volumetric flask, 5ml aliquots were taken and introduced in distillation unit through the side tube funnel. The glass stopper was immediately fitted. To this 10ml 40% NaOH into the digest. NH$_3$ is liberated into 10 ml 2 percent boric acid (with mixed indicator) containing 50ml conical flask. After distillation green coloured ammonium borate was titrated against 0.035NHCl till the end point (faint pink) was obtained (This gave 1ml 0.035NHCl = 0.5mg N% crude protein = %N x 6.25). Crude protein of seeds was calculated as percent nitrogen liberated x 6.5.

**Estimation of reducing sugar:**

The sugar content in the plant material was estimated by the procedure recommended by Oser (1979) as follows.

500mg of seed powder was taken in 50ml distilled water and boiled it, then filtered it and the filtrate is diluted upto 100ml. Three Folin-wn tube were taken and added following manner. (1) Blank tube – D.W. 2ml (2) 2ml glucose ‘C’ solution. (3) 2ml filtrate. In each tube 3ml alkaline solution of copper was added. Then tube was boiled in boiling water bath for 8 minutes. Cooled the tubes under tap water and add 2ml of phosphomolybdic
acid solution which give blue colour. Then this solution was diluted upto 25ml distilled water and optical density determined at 420nm and calculate the amount of reducing sugar present in seed powder.

**Estimation of crude fibre:**

Crude fibre (CF) is determined as that fraction remaining after digestion with dilute solutions of sulphuric acid ($H_2SO_4$) and sodium hydroxide (NaOH) under carefully controlled conditions. The major part of it contain carbohydrates and it is valuable parameter in deciding the nutritive quality of animal feed (A.O.A.C; 1970).

2g seed powder was taken in a 500ml spoutless beaker and added 200ml 1.25% $H_2SO_4$ to it. Break up the lumps with the help of glass rod having a rubber policeman. Cover the beaker with a conical flask, half filled with cold water, which servers as water condenser. Boiled for 30 minutes made up any loss in volume during the boiling with hot distilled water. Then filter through Whatman filter paper No.54 by washing the residue several times with hot distilled water. Take out the residue back in the beaker with 100ml water and to it added 100ml 2.5% NaOH. Boiled for 30minutes as earlier. Filtered through previously weighed Whatman filter paper No.54. Washed the residue several times with hot water and lastly with 70% alcohol. Dried it over night at 100°C to a constant weight, cooled and weigh. Incinerate the residue along with filter paper in a crucible at 600±20°C for 2hrs in a muffle furnace until all the carbonaceous matter is
burnt. Cooled the crucible in a descicator and weigh. Recorded the loss in weight as crude fibre.

**Estimation of calcium:**

An aliquat (25ml) of the acid solution ash portion was diluted to about 150ml with distilled water. Few drops of methyl red are added and the mixture is neutralised with ammonia (NH₃) solution till the pink colour changes to yellow. The solution was heated to boiling and the 10ml ammonium oxalate solution was added. The mixture was allow to boiled for a few minutes. Glacial acetic acid was then added till distinctly pink colour reappeared. The mixture was then kept aside for 12 to 24 hours at room temperature. When the precipitate at calcium oxalate settles down, it was filter through Whatman filter paper No.42. The precipitate was washed several times with water, to make it free from acid. It was then transferred in a small beaker by piercing a hole in the filter paper and by pouring over it about 15ml 2N H₂SO₄. This is heated to above 40ºC and titrated against 0.01N KMnO₄ solution until the first drop which gives the solution a pink colouration persisting for atleast 30 second. The amount of calcium was calculated using an equation. 1ml of KMnO₄=0.2004mg of Ca. The percent Ca on DM basis was then calculated on the basis of the amount of sample used for ashing, the volume to which acid solution of ash is diluted and the volume of the aliquat taken for the precipitation of calcium. The procedure of estimation of calcium is recommended by A.O.A.C. (1970).
Estimation of phosphorus:

The estimation of phosphorus is carried out by the method given by Fiske and Subba Row (1925) and recommended by Oser (1979).

0.5ml of acid soluble portion of ash was taken in a test tube. Diluted it to a volume of 10ml with distilled water. Simultaneously taken a blank containing only 10ml distilled water. Added 1ml molybdate solution to each test tube and mix, then added 0.4ml ANSA reagent and again mix. Allowed to stand for 5 minutes and noted/observed the optical density (O.D.) at 660μ using colorimeter by setting it to a zero with the blank.

Established the O.D. of standard phosphorus solution by preparing a standard graph containing 0 to 1ml standard phosphorus solutions in series of test tubes. Determinated the amount of phosphorus in an aliquot with the help of standard graph and calculated the phosphorus content in the seed powder considering its amount taken for ashing, volume of the acid soluble ash and amount of aliquot used for the reaction.

Estimation of Dry matter (DM):

Dry matter (DM) is calculated by weighing the sample after drying to a constant weight in an oven at 95±5°C. For this purpose, 100g of sample is taken in a clean dry pre-weighed tray and is kept in oven for 48 hours or more, till constant weight. Weight of the dried sample is reported as per cent dry matter (DM).

The dried samples are usually ground to a fine powder and stored in sealed container for further analysis A.O.A.C. (1970), Mungikar (1999).
Estimation of Nitrogen free extract (NFE) and total carbohydrates (TC):

Carbohydrates portion of biological material is made of two parts nitrogen free extracts (NFE) and crude fibre (CF). NFE is also known as soluble carbohydrate which consists of water soluble vitamins, monosaccharides (Pentoses and hexoses), oligosaccharides (compound sugars) and polysaccharides (starches). Insoluble carbohydrate or CF contain mainly polysaccharides consisting of hemicellulose and cellulose. The CF content of feed gives an indication of bulkiness of a feed. These two parameters are calculated by difference. NFE is represented (on DM basis) by a figure obtained when the sum of ash, protein, crude fat and crude fibre of a feed is substraction by from 100.

\[ \%\text{NFE} = 100 - (\% \text{CP} + \% \text{C Fat} + \% \text{CF} + \% \text{ash}) \]

Total carbohydrates (TC) is then determined as either

\[ \% \text{TC} = \% \text{NFE} + \% \text{CF} \]

OR

\[ \% \text{TC} = 100 - (\% \text{CP} + \% \text{C Fat} + \% \text{ash}) \]

Biological Control

1) Use of antagonistic microorganisms

Antagonistic potential of *Trichoderma viride* against pea fungi was studied by dual culture method used by Sudhamoy Mandal *et al.* (1999). An agar disc (5mm) containing mycelium of *Trichoderma viride* was inoculated at the centre of Czapek’s poured petriplates and culture discs of the test fungi were placed at the centre of the plate. Petriplates were
incubated for a week at 25±1°C. Plates without antagonists served as control. Two replicates were kept for each treatment and observations on colony diameter (mm) and formation of inhibition zone were recorded.

2) **Use of plant extracts**

Fungitoxicity of plant extracts was studied by the poisoned food technique described by Nene and Thapliyal (1993). Czapek dox agar medium was prepared in flasks and sterilized. To this medium, was added the requisite quantity of the plant extract was added. Plant extract was prepared by collecting fresh plant parts, washed thoroughly in distilled water and grinned in distilled water. The plant extract was thoroughly mixed by stirring. The medium was then poured into petriplates. Small disc (0.7 cm) of the fungus culture grown on potato dextrose agar for 7 days was cut with a sterile cork borer and transferred aseptically in the centre of the petridish containing the plant extracts. Suitable checks were kept where the culture discs were grown under the same conditions on Czapek’s dox agar without plant extract. Linear growth of the test fungi was measure at regular interval. The fungus colony diameter compared with check, was taken as a measure of the fungitoxicity.

The use of essential for biocontrol of fungi, the same method was implemented were the plant extract was replaced by pure essential oil.