Chapter III

MATERIALS AND METHODS

3.1 Experimental site:

The present study was carried out at the Biocontrol Laboratory, Department of Plant Pathology, Chandra Shekhar Azad University of Agriculture and Technology, Kanpur, Uttar Pradesh–208002, India (North East Plains Zone, India, 26°29′35″N and 80°18′35″E at an altitude of 125.9 m above the mean sea level). Soil samples were collected and native *Trichoderma* sp. were isolated from rhizospheric soil of pigeonpea fields in different districts of Uttar Pradesh.

3.2 Source of chemicals:

Chemical used in this isolation and identification procedure were purchased from Merck, Genie, Hi-media and SRL. The list of items used in experiments, i.e., glassware, plasticware and equipments is provided in Appendix-I and composition of media and reagents is provided in Appendix-II.

3.3 Sterilization procedure:

3.3.1 Sterilization of glasswares

Glasswares were washed in liquid detergent under running tap water, and rinsed with distilled water 2-3 times. Contaminated and stained glassware were washed with chromic acid (20-30 g potassium dichromate + 1.01 g sulfuric acid) followed by rinsing with distilled water 2-3 times. These were air-dried and then kept in oven for sterilization at 180°C for at least 2 h. Plastic wares were autoclaved at 121°C, 15 psi for 15 min.

3.3.2 Sterilization of media and distilled water:

Sterilized glassware and plastic wares were used for dispensing media and distilled water. All media were autoclaved at 121°C, 15 psi pressure for 15-30 min.

3.3.3 Sterilization of laminar air flow:

Prior to the day of inoculation of soil sample, the laminar air flow chamber

was saturated with alcohol vapors. At the time of inoculation the laminar air flow chamber was wiped with 70% alcohol or general spirit. Then only required instruments were kept in the chamber and exposed to UV rays for 15-20 min. All the operation viz. transfer, inoculation etc. were done over a gas burner flame.

3.4. Collection of the diseased material

Diseased pigeon pea plants material showing characteristic wilt symptoms were collected from different locations of Uttar Pradesh during the months of November and December 2010 and brought to the laboratory for critical examination.

3.5 Symptomatology

Pigeon pea plant may show wilt infection throughout their life cycle, but these are most common in reproductive stage. The typical symptoms are drying of plant and wilting. The leaves show mild chlorosis and yellowing before wilting .Wilting plants produce light brown or purple streaks rising from the base upwards on the main stem. Browning or blacking of the wood is seen, if the bark from the streak area is peeled off. Cross- and longitudinal-sections of the wilted plants show brown to black discoloration of xylem vessels. Sometimes partial wilting is also observed which is due to lateral root infection.

3.6 Isolation, purification and morphological characterization of pathogen and bioagent

3.6.1. Isolation and purification of pathogen, *Fusarium udum*

For isolation of pathogen *Fusarium udum* wilt affected roots were taken and dust particles removed under laboratory conditions. The roots were cut into small pieces with a sterilized scalpel and surface sterilized with 0.1% mercuric chloride solution for one minute. Thorough washing was done in sterilized water for three times and excess water was removed using sterilized blotting paper. These dried root pieces were finally transferred on 2% potato dextrose agar (PDA) medium with the help of sterilized pointed forceps.

The petridishes used for isolation were sterilized in hot air oven at 160°C for two hours and poured with 2% PDA medium (autoclaved at 1.5 kg pressure/cm^2 for 15 or 20 min). The inoculated petridishes were then incubated at 25-28°C for the growth of fungus. After 20 to 30 h of incubation, the white mycelial growth appeared around the pieces placed in the petridishes.

The growing hyphal tips of the colonies in different petridishes were transferred into culture tubes containing 2% sterilized PDA medium. The culture was purified by single spore culture method of isolated fungus. A dilute suspension was poured on plain agar in petridish and spores were allowed to settle down on agar medium. The spores, which were settled quite apart from each others, were selected under microscope marked with help of inoculation needle. They were lifted along with agar and transferred to 10 petridishes containing 2% sterilized PDA medium and maintained at 10°C in incubator for further studies.

3.6.2 Isolation and purification of *Trichoderma* sp.

Soil samples were collected from various rhizospheric soils of pigeon pea field of different places of Uttar Pradesh. *Trichoderma* sp. isolated from collected soil samples with the help of serial dilution plate technique (**Johnson and Crul, 1972)** were grown on PDA medium for proper identification. 10 g well-pulverized air-dried soil sample was added in 90 ml sterile water in a flask making 1:10 dilution and shaken vigorously on a magnetic shaker for 20-30 min to obtain homogenous suspension. One ml of suspension from the flask was transferred into a test tube containing 9 ml sterile water under aseptic condition to make 1:100 dilutions. Further, dilution was made to 10^3 by pipetting 1 ml suspension into additional water as per above procedure. One ml of the suspension from 10^3 dilutions was transferred into 10 sterile petriplates containing 15 ml sterile PDA medium and spread uniformly. The inoculated petriplates were incubated at 23±2°C for 7 days in an incubator.

3.6.2.1 Identification of bioagent

The fungus was grown on PDA medium. The measurements of different

morphological structures were done under the microscope by slide mounting and on the basis of molecular analysis using ITS (internal transcribed spacer) marker. Observations of the following morphological characters of fungus were noted.

3.6.3 Mycelial characteristics of pathogen

Color, branching pattern and width of the hyphae, microconidia, conidiophores and chlamydospore were studied under microscope for identification.

Sporodochia: Its presence, color, shape, size and arrangement were recorded under the compound microscope.

Microconidia: Number of branching pattern, size, shape and structure of microconidia were observed under microscope for specific identification. These are produced on unbranched microconidiophores in dry small and false heads.

Macroconidia: Microscopic analysis of macroconidia number, color, size and arrangement was done for specific identification. They are produced on branched macroconidiophores, which were fucoids with pointed ends.

Chlamydospore: Studies on the position, arrangement, shape and morphology of chlamydospores were done for better understanding about the pathogen. Sometimes chlamydospores-like swellings were seen on the hyphae.

3.7 Cultural characteristics of bioagent *Trichoderma*:

3.7.1 Effect of different media on growth of bioagent *Trichoderma*

The following ten different natural and synthetic media were used to study the morphological and cultural characters of the bioagent *Trichoderma*. All the media were sterilized in an autoclave at 1.1 kg/ cm^2 pressure for 30 min at temperature of 121.6^0C.

***Trichoderma* Selective Medium (TSM) (per liter) (Appendix II):**

$MgSO_4.7H_2O$	0.2 g
K_2HPO_4	0.9 g

NH_4NO_3	1.0 g
KCl	0.15 g
Rose Bengal	0.15 g
Glucose	3 g
Agar	20 g

Trichoderma-selective agar medium (TSM) was developed for quantitative isolation of *Trichoderma* sp. from soil. Selectivity was obtained by using chloramphenicol (0.25 g/l), streptomycin (9.0 ml/litre) as a bacterial inhibitor, and pentachloronitrobenzene, *p*-dimethylaminobenzenediazo sodium sulfonate (1.2 ml/l) and Rose Bengal (0.2 g/l) as selective fungal inhibitors. TSM also contains a low concentration of glucose which still allows relatively rapid growth and sporulation of *Trichoderma,* enabling convenient and rapid identification of *Trichoderma* colonies **(Elad *et al*., 1981)**.

Potato Dextrose Medium (PDA) (per liter):

Pealed potato	200 g
Dextrose	20 g
Agar	20 g

Potato slices were cooked in 500 ml of water, filtered with a muslin cloth and then the volume was made up to 1,000 ml with water. Refiltration was done using muslin cloth. Dextrose was added to this mixture and shaken well.

Asthana and Hawkers Medium (per liter)

Potassium nitrate	3.05 g
Magnesium sulphate	0.75 g
Potassium dihydrogen phosphate	1.75 g
Glucose	5 g
Agar	15 g

Richard's Agar Medium (per liter)

Potassium nitrate	10 g
Potassium dihydrogen phosphate	5 g
Magnesium sulphate	2.5 g
Ferric chloride	0.2 g
Agar	15 g
Sucrose	500 g

Sabouraud's Agar Medium (per liter)

Glucose	40 g
Peptone	10 g
Agar	20 g

Rose Cookie Bengal Agar Medium (per liter)

Dextrose	10 g
Magnesium sulphate	30 g
Potassium dihydrogen phosphate	1.05 g
Rose Bengal	30 g
Agar	20 g

Cooke Rose Bengal Agar Base (per liter)

Papaic digest of soybean meal	5 g
Dextrose	10 g
Mono-potassium phosphate	1 g
Magnesium sulphate	0.5 g
Rose Bengal	0.035 g
Agar	20 g

Pikovskaya's Agar Medium (per liter)

Yeast extract	0.5 g
Dextrose	10 g
Calcium phosphate	5 g
Ammonium sulphate	0.5 g
Potassium chloride	0.2 g
Magnesium sulphate	0.1 g
Manganese sulphate	0.0001 g
Ferrous sulphate	0.0001 g
Agar	15 g

Beef Extract Agar Medium (per liter)

Peptic digest of animal tissue	10 g
Beef extract	3 g
Sodium chloride	5 g
Agar	15 g

A) ***Solid media study:***

Effects of different media on the growth and sporulation of the identified *Trichoderma* sp. were studied to determine the linear growth rate. Linear growth rate of the identified bioagents *T. harzianum, T. viride, T. atroviride, T. koningii, T. longibrachiatum,* and *T. virens* were determined. Twenty ml of each sterilized solid media was poured into 90 mm size petridishes. The plates were inoculated with 5 mm circle pieces of inoculum cut with the help of sterilized scalpel from 7 d old culture in three replicates. Pure culture of *Trichoderma* (5 mm diameter) maintained by sterile cork borer were placed in the centre of the sterilized petridish containing different media separately in equal amounts (10 ml) and incubated at

25 ± 2^0C. The radial hyphal growth was observed in two directions at right angles to each other in first 48 h and then after every 24 h and average of the readings in three replicates were measured. The important colony characters like change in the colony color, rate of growth and sporulation and nature of colony were recorded and then, average was taken for statistical analysis.

B) ***Liquid media study****:*

Studies were carried out to determine the amount of mycelial mat produced from the identified bioagent *Trichoderma* sp. in five different liquid media including Potato Dextrose (PD broth), Rose Bengal, Malt, Sabouraud and Czapek dox Broth. Fifty ml of each liquid media were poured into 150 ml conical flasks in three replications and sterilized in an autoclaved at 1.1 kg/cm^2 pressure for 20 min. The flasks were incubated with 5 mm cut piece of the culture of *Trichoderma* sp. with the help of sterilized cork borer from 7 d old culture and placed in the incubator for 10 days at 25 ± 2^0C. At the incubation period, the contents of the flask were filtered through Whatman filter paper. Mycelial mats on filter paper were washed thoroughly with distilled water and then dried at 60^0C for 72 h, subsequently cooled in desiccators and weighed on electronic balance. Average dry weight of the mycelial mat of three replications of the bioagent was taken as standard value for comparison of growth under different treatments.

3.7.2 Effect of temperature on growth of bioagent:

To find out optimum as well as suitable temperature for the growth of biocontrol agent, *Trichoderma* sp. were grown at 5 different temperatures on PDA medium. For each temperature and pathogen 10 replicates were inoculated with a small amount of pure culture of *Trichoderma*. The inoculated petriplates were kept at 15, 20, 25, 30 and 35^0C in BOD incubator. The temperature is an important parameter for the growth of pathogen and biocontrol agent because it affects spore germination, mycelial growth and competitive saprophytic ability. After 10 days of inoculation, the mycelium mat was filtered through Whatman filter paper and dried in hot air oven at 60^0C, cooled in desiccators and weighed.

3.7.3 Effect of pH on growth and sporulation of the bioagent *Trichoderma* sp:

To find out optimum as well as suitable pH for the growth and sporulation of bioagent *Trichoderma* sp. was grown at 10 different pH values on dextrose broth medium. pH value plays an important role for growth and spore germination. The set of different pH values (4, 4.5, 5, 5.5, 6, 6.5, 7, 7.5, 8 and 8.5) was prepared and pH was adjusted by adding appropriate amount of citrate phosphate buffer. For each pH value, there were three replications. The spore count was recorded by using haemocytometer as 1 x 10^5 spore/ml. One ml of this spore suspension was then added in PD broth of different pH ranges and then kept in BOD incubator at 23 ± 2^0C for 7 days. After 7 days, mycelium mat was harvested from each flask by collecting the culture filtrate through sterilized Whatman filter paper. The harvested mycelium as kept in hot air oven at 80^0C for 48h and weight was measured in mg.

3.8 Laboratory screening of antagonists against the test pathogen:

3.8.1 Evaluation of bioagents against the pathogen:

For this study, the pathogen was isolated from the diseased plant of pigeon pea field from different places of UP area. One week old culture of *Fusarium udum* maintained on PDA petriplates at 28 ± 1^0C was used for the study of the effects of biocontrol agents isolated from the rhizosphere of wilt sick plot. Antagonistic activity of these biocontrol agents against test pathogen was determined by "dual culture" technique. Five mm disc of pathogen was taken from the actively growing colonies of the test pathogen and antagonists with the help of inoculating tools. The disc of the pathogen was placed on one side of in agar plates aseptically, and the antagonists were placed opposite side of the pathogen in same petriplates. Each treatment was replicated three times and incubated at 28 ± 1^0C. Growth of bioagent and pathogen were recorded after 10 d of incubation. When the inhibition zone was formed, it was expressed as % inhibition and was calculated by the following formula:

$$\text{Inhibition \%} = \frac{C - T}{C} \times 100$$

where,

C = Growth of the colony in control (mm)

T = Growth of the colony in treated plates.

The biocontrol agents used for testing were:

(1) *Trichoderma viride*

(2) *Trichoderma harzianum*

(3) *Trichoderma longibrachiatum*

(4) *Trichoderma atroviride*

(5) *Trichoderma virens*

(6) *Trichoderma koningii*

3.9 Induction of xylanase from *Trichoderma* sp.:

3.9.1 Materials for xylanase induction

Glucose, maltose, carboxymethyl cellulose (CMC), sucrose, corn-cobs, wheat bran, oat spelt xylan and birchwood xylan were taken from Sigma Chemical Co., India. All the other chemicals used were of analytical grade.

3.9.2 Sterilization

Media, solutions and glassware were sterilized by autoclaving at 121.6^0C for 15 to 30 min. When high temperature was not recommended, sterilization of solutions was performed by filtration through sterile 0.2 mm pore size cellulose acetate membrane filters. Glassware was baked overnight in an oven at 200^0C.

3.9.3 Maintenance of *Trichoderma* sp. culture

Isolates of *Trichoderma* sp. were maintained at 4^0C after growing for 7 d in MYG medium (10.2% malt extract, 0.2% yeast extract, 2% glucose and 2% agar) at 28^0C (**Azin *et. al*., 2007).**

3.9.4 Sporulation medium used for *Trichoderma* sp.

The sporulation medium used for the fungus is given in table:

Table 3.1: Composition of sporulation medium for *Trichoderma* sp.

Ingredients	Quantity (g/100 ml)
Trisodium citrate	0.5
KH_2PO_4	0.5
NH_4NO_3	0.2
$(NH4)_2\ SO_4$	0.4
$MgSO_4$	0.02
Peptone	0.1
Yeast Extract	0.2
Glucose	0.2
Agar	2.5
Distilled H_2O	To make volume up to 100 ml

3.9.5 Inoculum preparation

Inoculum (100 ml) was prepared similarly as given in Table 3.2 except that agar was not added to the medium. Glass beads were used for uniform growth. Its pH was adjusted to 5.5 with 1 N NaOH/ HCl. Loopful culture of *Trichoderma* sp. were taken for sporulation.

3.9.6 Enzyme production (EP) medium

The fungus was grown in EP medium for xylanase production.

Table 3.2: Composition of enzyme production medium

Ingredients	**Quantity (g/l)**
Glucose	3.0
Bactopeptone	1.0
Urea	0.3
$(NH_4)_2SO_4$	1.4
$MgSO_4$. $7H_2O$	0.3
$CaCl_2.6H_2O$	0.3
Xylan	2.0
Trace element solution (Fe^2+Mn^2+Zn^2+CO^2)	0.1%

Trichoderma sp. was then grown modified Vogel's medium for xylanase production. Erlenmeyer flasks (500 ml) containing 100 ml Vogel's medium were plugged with cotton. The pH of the medium was adjusted to 5.5. Inoculum (10 ml) was added to each medium under laminar air-flow. The flasks were incubated on rotary shaker at 150 rpm at 28^0C for 5 days. The composition of trace element solution used in modified Vogel's medium (Table 3.3) in g/100 ml.

Table 3.3: Modified Vogel's medium for xylanase production.

Chemical	**Quantity (g/100 ml)**
Trisodium citrate	0.5
KH_2PO_4	0.5
NH_4NO_3	0.2
$(NH_4)_2SO_4$	0.4
$MgSO_4$	0.02
Yeast extract	0.5

Xylan	1
Tween 80	50 µl
Trace element solution	1 ml

Trace elements	**Quantity per ml**
Citric acid	5.0 g
$ZnSO_4.7H_2O$	5.0 g
Fe $(NH_4)_2$ SO_4	1.0 g
$CuSO_4.5H_2O$	0.25 g
$MnSO_4.H_2O$	0.05 g
H_3BO_3.anhydrous	0.05 g
$Na_2MoO_4.2H_2O$	0.05 g

3.9.7 Harvesting of cultures

Liquid state cultures were harvested by centrifugation at 10,000 rpm for 20 min at 4^0C, and the resulting supernatant was considered as crude enzyme preparation.

3.9.8 Xylanase activity

Activity of the enzyme was performed by the method described by **Khanna and Gauri (1993)** using a 0.1 M citrate buffer with pH 5.0.

3.9.8.1. Reagents

(A) Citrate phosphate buffer

24.3 ml of 0.1 M citric acid and 25.7 ml of 0.2 M disodium phosphate (Na_2HPO_4) were mixed with magnetic stirrer and pH was adjusted to 5.0 by citric acid or Na_2HPO_4. After adjusting pH the volume was made to 100 ml with distilled H_2O.

(B) Dinitrosalicylate reagent (DNS) (per liter)

Na.K tartarate	128 g
NaOH	10 g
DNS	10 g
Phenol	2 g
Na_2SO_4	0.5 g

3.9.8.2. Assay of xylanase activity

Xylanase activity was assayed using 1% (w/v) of birch wood xylan as a substrate. Reaction mixture contained 1ml of appropriately diluted enzyme and 1% xylan in citrate phosphate buffer. The mixture was incubated at 50ºC for 30 min. The reaction was terminated by adding 3 ml dinitrosalysilic acid (DNS) reagent **(Miller, 1959)**. After heating for 5 min in a boiling water bath and cooling, the absorbance was noted at 550 nm. Xylose was used as standard. One unit (IU) of enzyme activity is defined as the amount of enzyme required to liberate 1 μmole of xylose from the appropriate substrates under the standard conditions.

3.9.9 Effect of various carbon sources on xylanase activity

Trichoderma sp. was grown in modified Vogel's medium with different carbon sources like glucose, maltose, CMC, sucrose, corn-cobs, wheat bran, oat spelt xylan and birch wood xylan to check their effects on the expression of xylanase.

3.9.10 Effect of pH on xylanase activity

The activity of the crude xylanase was measured at pH ranging from 4.0 to 8.0 using different buffers. Sodium acetate buffer was used for pH 4-5.5 and citrate phosphate buffer was used for pH 6-8.

3.9.11 Effect of temperature on xylanase activity

The reactions were carried out at 40, 50, 60 and 70ºC for determination of optimum temperature for xylanase activity.

3.10. Biochemical analysis (Protein estimation)

3.10.1 Protein estimation through Kjeldahl method:

Total protein content of different isolates of bioagent was estimated by Kelplus nitrogen analyzer. Total protein nitrogen was estimated by this method was multiplied by a factor for estimating the total protein content.

Requirements:

(1) Digestion mixture (mixed potassium sulphate with copper sulphate in the ratio of 9:1)

(2) Boric acid 4% (dissolved 40 g H_3BO_3 in 1 liter of distilled water)

(3) 40% acidic NaOH solution

(4) N/10 HCl solution (diluted 9 ml of conc. HCl to 1 liter with distilled water)

(5) Mixed indicator dye (added methyl red 0.2 g/100 ml ethanol and methylene blue 0.2 g/100 ml ethanol in the ratio 1:1)

Procedure:

100 mg dried and well-powdered sample was accurately weighed on a piece of filter paper and transferred along with the filter paper to 30 ml micro Kjeldahl digestion tube. Then 3 ml of conc. H_2SO_4 and 500 mg digestion mixture were added to digestion tube. Sample was digested on an electric heater at 400^0C for one hour.

After cooling, the digest was transferred to micro Kjeldahl distillation apparatus using successive small quantities of water. 10 ml of 40% NaOH solution was poured in it and NH_3 liberated by steam distillation was collected in 100ml conical flask containing 10 ml of 4% boric acid solution in which few drops of mixed indicator was added. Boric acid containing ammonium borate was titrated against N/10 standard HCl until the first appearance of violet color at the end point. A reagent blank with filter paper (no sample, only digestion mixture and H_2SO_4 was taken) also run and titrated value for blank was also recorded. Percent

nitrogen in the sample was calculated by using following formula:

Nitrogen % (in 100 g) = [(1.4 x N x V)/W] x 100

Protein % = Nitrogen % x 6.25

where,

N = Normality of HCl

V = Titer value of sample – titer value of blank

W = Weight of the sample

3.10.2. Protein profiling of bioagent through SDS-PAGE:

Xylanase enzyme crude extract was purified by ammonium sulfate fractionation (80% saturation) and fast protein liquid chromatography (FPLC) and subjected to sodium dodecyl polyacrylamide gel electrophoresis as per the protocol given by **Sambrook and Russell (2001)**.

3.10.2.1. Materials required for SDS-PAGE:

To pour gels

30% Acrylamide bisacrylamide solution

10% SDS

10% APS (made fresh each time)

20 µl N, N, N', N' Tetramethylethylenediamine (TEMED)

1.5 M Tris, pH 8.8 (resolving gel)

1.0 M Tris, pH 6.8 (stacking gel)

5 X SDS running buffer (1 liter)

Tris	15 g
Glycine	72 g
SDS	5 g

Coomassie Blue stain R250

Acetic acid	10% (v/v)
Coomassie blue dye	0.0006% (w/v)
H_2O	90 ml

Isopropanol fixing solution

Acetic acid	10% (v/v)
Isopropanol	25% (w/v)
H_2O	65 ml

SDS sample loading buffer (40 ml)

H_2O	16 ml
0.5 M Tris, pH 6.8	5 ml
50% Glycerol	8 ml
10% SDS	8 ml
β-mercaptoethanol	2 ml (added immediately before use)
Bromophenol	10% (w/v)

Destaining of gel

Destain solution (20% methanol, 10% acetic acid)

Other reagents:

200 ml methanol

100 ml glacial acetic acid

10% (w/v) Trichloroacetic acid (TCA)

700 ml double distilled water

3.10.2.2. Procedure for SDS-PAGE:

The SDS-PAGE involved following steps:

A) Preparation of sodium dodecyl polyacrylamide gel

The glass plates were assembled as per the manufacturer's instruction. 17 ml of 12% polyacrylamide gel was poured into the space between the glass plates up to a level leaving sufficient space for the stacking gel. 0.1% SDS was poured on the top of the polyacrylamide gel in order to make the surface smooth and level. The gel was left until it solidified. After solidification, SDS layer was removed by washing with water for two to three times for polymerization, after that comb was carefully removed and wells were washed with deionized water two to three times to remove the unpolymerized acrylamide. While pouring the resolving gel and stacking gel, enough care was taken to avoid air bubble formation in the gel.

B) Preparation of sample for SDS-PAGE

The sample for the SDS PAGE was prepared as described above and equal volume of the loading dye was added and heated up to 95^{o}C for 10 min. To each well about 50 µg of the sample and equal volume of dye (w/v) mixture was loaded.

C) Gel electrophoresis

After loading the sample, the apparatus was submerged in running buffer and rest of the parts of electrophoretic apparatus was assembled as per the manufacturer's instruction. Initially the voltage was kept at 75 mV. When protein entered the resolving gel the voltage was increased up to 120 mV. The gel was run up to the end of the resolving gel for four to five hours. After sufficient run, the glass plates were carefully removed and gel was transferred to tray containing the staining solution.

D) Staining and destaining of polyacrylamide gel

After electrophoresis, the gel was carefully removed and placed in the staining solution. The gel was stained for 3 h under shaking condition. After staining with coomassie brilliant blue R250, excess dye was removed by

destaining solution under shaking condition. The destaining solution was/ replaced two to three times until the clear blue color bands appeared. The gel was sealed in polyethylene bag and stored at 4^0C.

E) Fixing and staining

When electrophoresis was complete, the cassette was removed from the unit and gel was taken out gently. It was then placed in a staining tray/box and incubated overnight in 10% TCA. After incubation it was washed thoroughly with distilled water to remove excess SDS, which might had been precipitated on the surface. Pour sufficient amount of distilled water when staining was complete and the gel was rinsed with distilled water. Destaining in in destain solution for overnight cleared the gel background, resulting in a better resolution.

3.10.2.3. Photography, evaluation and documentation

The gel was illuminated from below with diffused fluorescent light and photographed. The gel was placed over a transilluminator and bands were captured by GelDoc system.

3.11. Genetic variability analysis through RAPD and SSR

3.11.1. DNA isolation of *Trichoderma* isolate

Pure culture of the target fungus was grown in liquid PD broth medium for the preparation of mycelium mat. The total genomic DNA was extracted from isolate of *Trichoderma* based on Cetyl Trimethyl Ammonium Bromide (CTAB) extraction method described by **Doyle and Doyle (1990)** with minor modifications.

3.11.1.1. Reagents required for fungal DNA isolation and purification:

1. 1 M Tris Cl (pH 8.0) - (121.1 g Tris–Cl dissolved in 800 ml of doubled distilled water and pH adjusted to 8.0 using 0.1 N NaOH; volume made up to 1 liter and autoclaved).
2. 0.5 M EDTA (pH 8.0) - (186.1 g sodium salt of ethylene diamine tetraacetic acid (EDTA) dissolved in 800 ml of double distilled water and pH adjusted to 8.0 using NaOH pellets; volume made up to 1 liter and autoclaved).

3. 4 M NaCl - (233g sodium chloride dissolved in double distilled water and volume made up to 1liter and autoclaved).

4. 10% CTAB - (50 g CTAB dissolved in double distilled water and volume made up to 500 ml).

5. 24.1 Chloroform:isoamyl alcohol mixture

6. 3 M Na-acetate solution - (246.1 g sodium acetate dissolved in double distilled water, volume made up to 1 liter and autoclaved)

7. Isopropanol

8. 70% ethanol - (dilution made by sterile double distilled water; 30 ml added in 70 ml of ethanol)

9. Tris-EDTA buffer ($T_{10}E_1$ i.e. 10 mM Tris and 1 mM EDTA) - (1.21g Tris and 0.372g EDTA was dissolved in double distilled water and pH was adjusted to 8 with 1 N HCl. Volume made up to 1 l and autoclaved.

10. RNase – (dissolved in sterile double distilled water at 10 mg/ml; The solution was heated to dissolve and cooled to room temperature. The stocks were stored at -20^{0}C).

3.11.1.2. Procedure for DNA isolation:

Ten microliter of the genomic DNA was analyzed by submarine gel electrophoresis using 1.0% agarose with ethidium bromide and the genomic DNA was visualized under Gel doc/UV transilluminator.

1. The CTAB was dissolve completely only after NaCl was added and the solution was heated at 65^{0}C in water bath and stirred.

2. 0.2-0.5 g (dry weight) lyophilized pad was placed in 50 ml disposable centrifuge tube. The pad was broken with a spatula or glass rod and ~ 5 ml of 3 mm glass beads were added. Briefly the pad was powdered with vigorous shaking (without liquid nitrogen).

3. 10 ml (0.5 g pad) of CTAB extraction buffer and 150 µl of 20 mg/ml

proteinase K were added and gently mixed to wet the entire powdered pad (stirred with a glass rod, if necessary) and the tube was placed at 65^0C water bath for 30 min. While samples were incubated, glass hooks were made from 9 inch Pasteur pipettes, 1 per sample.

4. The tube was cooled briefly and an equal volume of chloroform isoamyl alcohol mixture (24:1) was added. It was then mixed by rocking on rocker platform for 5 min followed by spinning in centrifuge at 10,000 rpm for 10 min at 4^0 C.

5. The aqueous supernatant was transferred to a new 50 ml tube leaving behind the interface. The volume of the supernatant was noted. An equal volume of isopropanol (at room temperature) was added and mixed by gentle inversion. The high molecular weight DNA precipitated out upon mixing. DNA was spooled out with a glass hook and rinsed (still on the hook) in 4 ml of 70% ethanol in a test tube.

6. The DNA was dissolved through the hook by twirling gently in the 15 ml tube of TE buffer (100 µl). The DNA came out the hook within a few minutes. The re-suspended sample tubes were placed at 65^0C in water bath and the pellets were allowed to re-suspend overnight at 4^0C.

7. Genomic DNA was incubated at 37^0C for 30 min with 2 µl RNase (10 mg/ml).

8. After incubation, the sample was re-extracted with PCI solution (phenol: chloroform: Isoamyl alcohol 25:24:1) and RNA-free DNA was precipitated with chilled absolute ethanol.

3.11.1.3. Determination of the yield:

DNA concentration was determined by UV spectrophotometric analysis at 260 nm and quantitative analysis was done on 1% agarose gel.

3.11.1.4. Qualitative and quantitative estimation of DNA:

Quantification of DNA was done by analyzing the purified DNA on 1%

agarose gel. Quantified DNA was diluted in TE buffer to a concentration of approximately 12.5 ng/µl for use in PCR amplification. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.11.1.5. Agarose gel electrophoresis:

200 ml of 1% agarose gel was prepared. For making gel, 180 ml distilled water + 20 ml TAE buffer (10 X; 242 g Tris base, 57.1 glacial acetic acid and 100 ml 0.5 M Na_2EDTA for 1litre) + 2 g agarose was added and boiled to dissolve and then kept it for cooling. The comb in gel casting tray was fixed and agarose solution was poured slowly. It was kept for 30 min to solidify the gel.

3.11.1.6. Running of gel and visualization of DNA:

The comb was pulled out. 1 kbp DNA ladder (2 µl + 2 µl TAE) + 2 µl 6X loading dye [10 mM Tris-HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM Na_2EDTA] was loaded in first well, in subsequent wells the 6 µl each of isolated DNA samples were loaded. The gel was run in 1X TAE buffer at 60 V for 2 h. The gel was stained with ethidium bromide and with the help of gel documentation system (UVI Tek, UK), the gel was visualized and photograph was taken.

3.11.1.7. PCR amplification:

The PCR reactions were carried out in sterile 0.2 ml thin-walled PCR tubes obtained from Axygen Scientific Pvt. Ltd. USA.

RAPD and SSR amplification were carried out in 25 µl reaction volume containing sterile double distilled water (19.25 µl), 2.5 µl of 10X PCR assay buffer (final concentration 1X containing 50 M KCl, 10 mM Tris Cl, 1.5 mM $MgCl_2$), 0.25 µl dNTP mix (containing 250 µM each of dATP, dTTP, dGTP and dCTP), primer (20 ng) (1 µl of random decameric primers in case of RAPD and 0.5 µl random hexameric of each forward and reverse primer in case of SSR analysis), *Taq* DNA polymerase (stock 3 U/µl; 0.5 Units) and 2 µl template DNA (25 ng).

Amplification was performed in a master cycler with lid heating option at

105^0C with initial denaturation of genomic DNA at 95^0C for 2 min followed by 45 cycles of template denaturation at 94^0C for 1 min. Primer annealing was done at 36^0C for 45 sec, extension at 72^0C for 2 min and a final extension at 72^0C for 10 min**.** The PCR amplified product (5 µl) was visualized by electrophoresis on 1% agarose gel.

3.11.1.8. RAPD and SSR scoring and data analysis

The image of the gel electrophoresis was documented through gel documentation system and analysis software. All reproducible polymorphic bands were scored were analyzed following unweighted pair-group methods with arithmetic average (UPGMA) cluster analysis protocol and computed *in silico* into similar matrix using NTSYSpc (Numerical Taxonomy System Biostatics, version 2.11 W) **(Rohlf, 1993).**

The RAPD and SSR patterns of each isolate were evaluated, assigning character state; bands were manually scored 1 for presence and 0 for absence and the binary data used for statistical analysis using the software UPGMA 2.1. The size of the fragments (molecular weight in base pairs) was estimated by using 1 kbp ladder marker, which was run along with the amplified products. "1" indicated the presence of band in the gel and "0" indicated its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient and clusters were generated by making dendogram using the UPGMA algorithm in NTSYSpc software.

3.11.1.9. Analysis of internal transcribed spacer region

The internal transcribed spacer (ITS) regions of the rDNA repeat from the 3' end of the 18S and the 5' end of the 28S gene were amplified using the two primers, ITS 1 and ITS 4, which were synthesized by FastPCR software for designing specific primers as this tool provides many advantages in terms of primer properties such as calculating melting temperature, GC content, hairpin formation, self-annealing, primer quality, base count and PCR fragment size (in bp). The tool generates a list of primers first and then produces a list of primer pairs (forward

and reverse) simultaneously giving their melting temperatures and primer quality that eases the selection of primer pairs that can be used for the amplification of the DNA sequences. on the basis of conserved regions of the eukaryotic rRNA gene **(White *et al*., 1990).**

ITS 1 primer: (5'-TCTGTAGGTGAACCTGCGG-3')

ITS 4 primer: (5'- TCCTCCGCTTATTGATATGC-3')

The PCR amplification reactions were performed in a 50 µl PCR master mix containing 50 mM KCl, 20 mM Tris HCl (pH 8.4), 2.0 mM $MgCl_2$, 200 µM deoxyribonucleoside triphosphates (dNTPs), 0.2 mM of each universal ITS primer, 40 ng/µl of template DNA and 2.5/U of *Taq* DNA polymerase. The cycle parameters included an initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 1 min, primer annealing at 55°C for 2 min and primer extension at 72°C for 3 min and a final extension for 10 min at 72°C. Amplified products were separated on 1.2% agarose gel in TAE buffer, stained with ethidium bromide (1 ng/ml) and electrophoresis was carried out at 60 V for 3 h in 1X TAE buffer. One Kbp ladder (MBI, Fermentas) was used as a DNA size marker. The gel was observed under UV transilluminator.

3.11.1.10. Purification of PCR product

The desired bands (PCR products) were purified by Qiagen gel extraction kit using the following protocol **William *et al*., (1990) and Dodd *et al*., (2004).** The DNA band was excised from the agarose gel with a clean sharp scalpel. Then the gel slice was weighed in an Eppendorf tube. Then 3 volumes of buffer QG to 1 volume of gel (100 mg per 100 µl) were added. The mixture was then incubated at 50°C for 10 min. The gel was dissolved by vortexing the tube every 2 to 3 min during the incubation until the mixture color was uniformly yellow. Then added 1 gel volume of isopropanol to the sample and mixed. A QIAquick spin column was then placed in a 2 ml collection tube provided by the supplier. The sample was applied to the QIA quick column followed by centrifugation for one minute so that DNA was bound to the column. The flow-through was discarded and the QIAquick column

was placed back in the collection tube. After this added 0.75 ml of buffer PE to QIAquick column and centrifuged for 1 min for washing. The flow-through was again discarded and the QIAquick column centrifuged for an additional 1 min at 10,000 rpm. The QIAquick column was now placed into a clean 1.5 ml Eppendorf tube. Then added 50 µl of elution buffer (EB) (10 mM Tris-Cl, pH 8.5) to the center of the QIAquick membrane and centrifuged the column for 1 min to elute DNA.

3.11.1.11. DNA sequencing of the 18S rDNA fragment

The 28S rDNA amplified PCR product (100 ng concentration) was used for the sequencing with the single 18S rDNA 19F Forward, ITS 1 primer: 5'-TCTGTAGGTGAACCTGCGG-3' and 20R Reverse ITS 4 primer: 5'-TCCTCCGCTTATTGATATGC-3' synthesized by DNA Sequencer (Merck lab, Bangalore).

3.11.1.12. Sequence analysis

A comparison of the 18S rRNA gene sequence of the test strain against nucleotide collection (non-redundant/nucleotide) as a database was done using BLAST**.** A number of sequence of *Trichoderma* were selected on the basis of similarity score 95% of the determined sequence with a reference sequence. Multiple sequence alignment of these selected homologous sequences and 18S rRNA gene sequence of test strain was performed using ClustalW **(Thompson *et al.*, 1994)**. Subsequently, an evolutionary distance matrix was generated from these nucleotide sequences in the dataset. A phylogenetic tree was then drawn using the neighbor joining method **(Saitou and Nei, 1987)**. Phylogenetic and molecular evolutionary analyses were conducted using MEGA (Molecular Evolutionary Genetics analysis) version 4.0 **(Tamura *et al.*, 2007)**. The 18S rRNA gene sequence of test strain was again compared with different sets of sequence database such as small subunit ribosomal RNA (SSU rRNA) and large subunit ribosomal RNA (LSU rRNA) using Ribosomal RNA BLAST **(Altschul *et al.*, 1997).** 18S rRNA gene sequence of test strain was also compared against those

sequences in Ribosomal Database Project **(Cole *et al.*, 2009)** by using the RDP Classifier Check Program. The annotated information for the sequence in the database to which 18S rRNA aligned was used for fungal identification.

3.12. Validation of bioformulations under laboratory conditions (*in vitro*)

3.12.1. Preparation of bioformulations and determination of shelf-life:

Talc powder was evaluated as carrier material to produce bioformulation of *Trichoderma* sp. The carrier was dried under sun, powdered (sieve pore, 1 mm) and sterilized at 1.05 kg/cm^2 pressure for 30 min. The substrate was mixed with 7 d old culture of respective *Trichoderm*a sp. which were previously grown on PDA media in 2:1 (solid culture) w/v and CMC 5 g/kg was added as adjuvant. Fifty grams of such mixture was then filled in polypropylene bags (25 x 30 cm), tied and stored at 25±2^{0}C. Observations on colony forming units (cfu) of *Trichoderma* sp. were recorded initially and at monthly interval up to 6 months for shelf-life study.

The shelf-life of all the six isolates was also ascertained at ambient environment prevailing during a period of 6 months on the basis of spore load per gram. The talc-based powder of the bioagent was prepared (*Trichoderma* spore + mycelium) 1.0% w/w + Talc 98.5% w/w + 0.5% carboxyl methyl cellulose) were used for shelf-life, bioefficacy studies. The talc-based bioformulation was stored in low density polyethylene (LDPE) pouches. The powder was dull white in color, pH 7, moisture 8% and cfu of 29.7x10^6.

3.12.2. Testing of bioformulations:

All the treatments were tested by dry seed treatment method **(Nene and Thapliyal, 1977)**. This method was used to know the effect of seed-borne inoculum on seed quality parameters of pigeon pea, i.e., to carry out germination and vigor tests of apparently healthy and infected seed lots of pigeon pea and also to see the effect of different seed treatments on seed-borne inoculum as per the International Seed Testing Association Rules (**Anon, 1996**). Required quantity (5 g/kg of seed) of biocontrol agent along with 100 seeds of pigeon pea was used for

studies. Seed treatment was carried out with the help of paper towel method or rolling towel method **(Shailbaba and Tripathi, 2004).** Randomly selected 100 seeds were placed on two layers of moist germination paper, which were placed on a polythene paper and rolled carefully to avoid any excess pressure on seeds. These towels were incubated in seed germinator at 20±2ºC for 14 days. The first count was taken on fourteenth day. All morphologically normal seedlings were counted and germination was expressed in percentage.

To find out the seedling vigor, ten normal seedlings were taken from the germination test at random and the root length was measured from the collar region to the tip of the primary root and the mean root length was expressed in cm. The same seedlings were used for the measurement of shoot length. The shoot length was measured from the collar region to the point of junction of cotyledons. The mean shoot length was expressed in cm. Vigor index was calculated by two recommended methods, given by **Abdul Baki and Anderson (1973)**. These are:

Vigor Index I = Seed germination (%) × Seedling length (cm)

where, Seedling length = Shoot +Root length (cm)

Vigor index-II = Seed germination (%) x Dry weight (g)