CHAPTER II

MATERIALS 

AND

METHODS
2. MATERIALS AND METHODS

2.1 Test organisms:

Three different bacterial species and one fungal species were taken as test organisms. The bacterial species were tested for their antagonistic activities against the plant pathogenic fungi, *Tilletia indica*.

1. *Tilletia indica*

**Other Scientific Name:** *Neovossia indica* (Mitra) Mundk.

Domain: Eukaryota

Kingdom: Fungi

Phylum: Basidiomycota

Class: Ustilaginomycetes

Order: Tilletiales

Family: Tilletiaceae
Genus: *Tilletia*

Species: *indica*

*T. indica* is the fungal pathogen causing Karnal bunt of wheat. The fungus is almost impossible to eradicate from the soil as the teliospores (released from the fungus) remain viable for up to 5 years in infested soil. These teliospores are dark reddish to coppery, dull brown or dark brown in color, typically black/opaque, globose to subglobose, occasionally with a mycelial fragment (apiculus) attached; 24-47 µm diameter; exospores with thick, truncate, compact projections. The main host of *T. indica* is wheat (*Triticum* spp.) (Aujla *et al.*, 1989); durum wheat and triticale are less susceptible. It infects the plant within 2-3 weeks of heading.

2. *Cellulomonas* *spp.*

Domain: Prokaryota

Kingdom: Bacteria

Phylum: Actinobacteria

Class: Actiobacteria

Order: Actinomycetales
Family: Cellulomonadaceae

Genus: Cellulomonas

*Cellulomonas* is gram-positive rod-shaped bacteria. One of the main distinguishing features of these bacteria is their ability to degrade cellulose, using enzymes such as endoglucanase and exoglucanase (Glazer and Nikaido, 2007)

3. *Pseudomonas aeruginosa*

Domain: Prokaryota

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gamma proteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *aeruginosa*
Pseudomonas aeruginosa is gram-negative, rod-shaped, asporogenous, and mono-flagellated bacterium. It has ability to catabolize a wide range of organic molecules such as benzoate. It is an opportunistic human pathogen because it seldom infects healthy individuals.

4. Pseudomonas fluorescens MTCC 9768

Domain: Prokaryota

Kingdom: Bacteria

Phylum: Proteobacteria

Class: Gammaproteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: Pseudomonas

Species: fluorescens

Strain: MTCC 9768

These bacteria reside in plant’s rhizosphere and produce a variety of secondary metabolites including antibiotics against soil borne plant pathogens (Paulsen, I.T. et. al., 2005).

### 2.2 Maintenance of bacterial and fungal culture:

The bacterial strain of *P. fluorescens MTCC 9768* was obtained from IMTECH, Chandigarh and was maintained on King's medium B (King et al., 1954) at 25±2°C bacterial shaker incubator.

**King’s medium B (g/L) pH 7.2±0.2**

- Proteose peptone: 20g
- Dipotassium hydrogen phosphate: 1.5g
- Magnesium sulphate heptahydrate: 1.5g
- Glycerol: 15 ml
- Agar: 20g

The cultures of *Pseudomonas aeruginosa* and *Cellulomonas* spp. were obtained from Centre of Biotechnology, University of Allahabad and were maintained in Nutrient Medium at 25±2°C in bacterial shaker incubator.
**Nutrient Medium (g/L)) pH 7.2±0.2**

- Peptone 5g
- Sodium chloride 5g
- Beef extract 3g
- Agar 15g

The fungal strain of *T. indica* was collected from G.B. Pant University of Agriculture and Technology, Pantnagar and was maintained in Potato Dextrose Agar (PDA) at 21°C ±4 in BOD incubator.

**Potato Dextrose agar (g/L) pH 5.6±0.2**

- Potatoes, infusion 200g
- Dextrose 20g
- Agar 15g

All the growth mediums were prepared in double distilled water and were sterilized using autoclaved at 15 lbs pressure (121°C) for 15 min.
2.3 Growth study of bacterial culture:

The three bacteria: *P. aeruginosa*, *Cellulomonas spp.* and *P. fluorescens* MTCC 9768 were grown in Nutrient broth and Kings B broth at optimal growth temperature in bacterial shaker incubator for 96 hr. After the interval of 24hr, 48hr, 72 hr and 96hr, 2 ml bacterial cultures were withdrawn from each flask in sterile condition. The growth of bacteria was recorded by measuring the absorbance at 560 nm using UV-visible spectrophotometer (*Pharmacia Biotech Ultrospec 4000 UV/Visible Spectrophotometer*, Britain). All data are represented as mean and standard errors (mean±S.E.). For the growth study, the variables were analyzed using two-way analysis of variance (ANOVA).

2.4 *In vitro* antifungal assays:

In order to test for antagonism, pairings were made between *P. fluorescens* MTCC 9768, *P. aeruginosa* and *Cellulomonas spp.* and *T. indica* using Dual culture technique (Fokkema, 1978). In this method, agar disc of 4 mm size in diameter was taken from the *T. indica* stock culture maintained on PDA and transferred on the center of test PDA plates. Two parallel bacterial streaks at 3 cm distance from the centre (*T. indica* disc) were made. The plate with only fungal culture disc without bacteria was taken as control. These plates were incubated at 25°C in BOD incubator for 14 days. Three replicates were made of each pairing and the
experiment was repeated twice. Degree of antagonism was determined by measuring the mycelia growth of the pathogen and inhibition zone after 72 hr of incubation. All data are represented as mean and standard errors (mean±S.E.). For the percentage of inhibition, the variables were analyzed using two-way analysis of variance (ANOVA).

In Agar plug diffusion method (Jiménez-Esquilín and Roane, 2005, Elleuch et. al., 2010), the three bacterial cultures were inoculated and spread over the entire surface of separate agar plates. Then, the agar disc of 4 mm size in diameter was taken from the *T. indica* stock culture and transferred on the center of test plates. The plate with only fungal culture disc without bacteria was taken as control. These plates were incubated at 25°C in BOD incubator for 14 days. Three replicates were made of each plate and the experiment was repeated twice. The degree of antagonism is tested by the growth of fungus in presence of bacteria.

The other *in vitro* antagonism tests are as follows:

**2.4.1 Minimum Inhibitory Concentration (MICs):**

To analyze the efficacy of antagonism, different concentration of $10^1 - 10^7$ cells ml$^{-1}$ (OD$_{600}$ nm from 0.5 to 2.0) of *P. fluorescens* MTCC 9768, *P. aeruginosa* and *Cellulomonas sp.* were inoculated and spread on modified agar medium along with fungal disc of 4 mm in diameter at the center of each plate and incubated in
BOD incubator at 25 °C for 14 days. The percentage of mycelial growth inhibition was calculated on 7th and 14th day of experiment by the equation given (Riungu et al., 2008):

\[
Percentage\ of\ Inhibition = \left(\frac{C - T}{C}\right) \times 100
\]

Where, \(C\) = Radial growth of fungus in control plates (mm) and \(T\) = radial growth of fungus on the plate inoculated with antagonist (mm).

All data are represented as mean and standard errors (mean±S.E.). For the MIC, the variables were analyzed using two-way analysis of variance (ANOVA).

2.4.2 Biomass estimation:

The biomass estimation was measured by calculating the fresh weigh of fungal mycelia. The three bacteria (\(P.\ fluorescens\) MTCC 9768, \(P.\ aeruginosa\), \(Cellulomonas\ sp.) were grown in King’s medium B broth in 100 ml flasks separately. The fungal (\(T.\ indica\)) discs were then suspended in the growth medium. The flasks were incubated in BOD shaker incubator at 25°C for 14 days at 100 rpm.

After the incubation period, on 7th and 14th day, the fresh mycelia were extracted and filtered using Whatmann filter paper and were weighed on digital weighing machine. All data are represented as mean and standard errors
(mean±S.E.). For the biomass estimation, the variables were analyzed using one-way analysis of variance (ANOVA).

**2.4.3 Protease assay using tyrosine:**

For the estimation of protease assay, the harvesting of fungal mycelia was done by scrapping the fungal mycelia from the test PDA and control plates and then storing at -20°C for 4-5 days. The protease enzyme was extracted using modified TCA- Acetone method (Natarajan et. al., 2005). The dried mycelia were powdered by crushing with liquid nitrogen in a mortar and pestle. The mycelia powders were suspended in 15 ml of 10% (w/v) TCA in acetone containing 0.07% (v/v) 2- mercaptoethanol. The mixers were vortexed and incubated in -20°C for 1 hr. The total protease was recovered by centrifugation the mixture at 14,000 rpm for 20 min at 4°C. The pellets were washed in acetone containing 0.07% (v/v) 2-merceptoethanol. The pellets were dried for 30 min and re-suspended in 1ml lysis buffer for sonication (4 cycle’s at pulse of 30 sec).

**Fungal lysis buffer:**

10mM phosphate buffer (pH 7.4), 9M Urea, 1% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1% pH gradient ( buffer 4-6), 1% DTT
The bacterial protease was isolated from the three bacterial cultures by centrifugation at 10,000 rpm for 15 min. The pellets were collected and mixed with protein lysis buffer and sonicated (4 cycle’s pulse of 30 sec). The ruptured cell lysates of fungus and bacteria were centrifuged at 13,000 rpm for 5 min and the supernatant were stored at -20 °C till use.

**Bacterial lysis buffer:**

50mM Tris (pH 8.0), 10% glycerol, 0.1% triton-X 100, 100µg/ml lysozyme, 1mM PMSF, DNase 3U + 2mM MgCl to the final conc.

The enzyme extracts were assayed for protease activity by the modified method of Anson, 1938. The enzyme isolates from *T. indica* alone and interacted with *P. fluorescens* MTCC 9768, *P. aeruginosa* and *Cellulomonas* sp. were collected in separate eppendorf tubes. In each tube, 150 ml of 10 mM CaCl$_2$ was added followed by the addition of 250 ml of 50 mM, Tris– HCl (pH 7.5) and 250 ml of 0.12 % gelatin (substrate). All the tubes were shaken and incubated at 37°C for 4 hrs. After incubation, the reaction was stopped by adding 1 ml of TCA (10 %) containing 0.22 M acetic acid and 0.33 M sodium acetate to each tube and incubated at 48°C for 1 hr. After the second incubation, all samples were centrifuged at 10,000 rpm at 48°C for 10 min. The concentration of tyrosine in the supernatant was determined by the Lowry method (Lowry *et. al.*, 1951) and
comparing with the tyrosine standard curve. All data are represented as mean and standard errors (mean±S.E.). For the specific activity of protease, the variables were analyzed using one-way analysis of variance (ANOVA).

One unit enzyme activity was defined as the amount of enzyme that releases 1 μg of tyrosine per ml per min. Specific enzyme activity was expressed as unit/mg of protein (Rangeshwaran and Prasad, 2000).

2.5 Morphological studies:

The morphological studies of the antagonistic effect of the three bacteria: *P. fluorescens* MTCC 9768, *P. aeruginosa* and *Cellulomonas sp.* on *T. indica* control was done using Lacto phenol cotton blue staining. The changes in the ultra structure of the mycelial were further observed under SEM.

2.5.1 Lacto phenol cotton blue staining:

The lactophenol cotton blue staining is done for the fungal cultures.

**Lactophenol cotton blue stain (g/L):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenol crystals</td>
<td>20g</td>
</tr>
<tr>
<td>Cotton blue</td>
<td>0.05g</td>
</tr>
<tr>
<td>Lactic acid</td>
<td>20ml</td>
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</tbody>
</table>
Glycerol 

Distilled water 

One drop of lactophenol cotton blue stain was placed on each of clean and dry glass slides. A small tuft of fungus (preferably with spore and mycelia) each from the *T. indica* control plate and *T. indica* interacted with the three bacteria was transferred into the drop using flamed cooled needle on separate glass slides. The material on slide was mixed well and a cover slip was placed onto it avoiding formation of air bubble. The mounted glass slides were observed under Inverted microscope (Leica DMi8) in Zoology Department, University of Allahabad.

2.5.2 Scanning Electron microscopy:

The SEM analysis was performed at University Science Instrumentation Centre (USIC), Babasaheb Bhimrao Ambedkar University, Lucknow. The dried fungal mycelia of *T. indica* (control) and *T. indica* interacted with antagonistic bacteria *P. fluorescens* MTCC 9768 was placed on the double-stick, electrically-conductive carbon tape coated with electrically conductive material. The samples were observed under *Jeol scanning electron Microscope (SEM) JEOL JAPAN JSM 6610 LV.*
2.6 Enzymatic antagonism assays of *P. fluorescens* MTCC-9768:

The enzymatic assays to show the antagonistic activity of *P. fluorescens* MTCC 9768 were performed. These enzymatic assays includes: Production of HCN, Siderophore, Cellulolytic, Protease, β-1,4 glucanase and chitinase.

2.6.1 HCN production:

The production of HCN was determined using modified method of Miller R.L., Higgins V.J. (1970). The antagonist bacterium (*P. fluorescens* MTCC 9768) was grown on Tryptic-soy-agar (TSA) supplemented with glycine (4.4 g/L).

**Tryptic-Soy-agar (g/L):**

- Animal peptone 5g
- Soy peptone 5g
- Sodium chloride 5g

The sterile paper discs soaked in a picric acid solution (2.5% picric acid and 12.5% sodium carbonate) were placed in the lid of petri-plates and sealed with parafilm. The plates were incubated at 28°C for 48 hr. Three replicates were made of each plate and the experiment was repeated twice. A change of the disc color
from yellow to light brown, brown or reddish brown was recorded as an indication of weak, moderate or strong production of HCN.

2.6.2 Siderophore production:

The bacterial culture of *P. fluorescens* MTCC 9768 was initially grown on King’s medium B in presence and absence of FeCl₃ (100 µM) (Radheshyam, K. *et al.*, 1990). An agar disc of 4 mm size in diameter was taken from the *T. indica* stock culture maintained on PDA and transferred on the center of test plates. The plates with fungal disc alone were used as control. The plated were sealed with parafilm and incubated at 28°C in BOD incubator. Three replicates were made of each plate and the experiment was repeated twice. The change of color from brown to purple indicated the presence of siderophore.

2.6.3 Cellulolytic assay:

Congo red clearing zone assay is suitable for qualitative analysis of Cellulolytic activity (Ariffin *et. al.*, 2006). The CMC agar plates were inoculated with the antagonistic bacterium culture (*P. fluorescens* MTCC 9768) and incubated at 37°C for overnight.

**CMC agar (g/l):**

Potassium dihydrogen phosphate 1.0g
Magnesium Sulfate Heptahydrate 0.5g
Sodium Chloride 0.5g
Iron (II) Sulfate Heptahydrate 0.01g
Manganese (II) sulfate monohydrate 0.01g
Ammonium nitrate 0.3g
Carboxymethyl Cellulase (CMC) 10.0g
Agar 20.0g

After incubation, the plates were flooded with 0.1% congo red solution and left for 15 min. Thereafter, the plates were flooded with water and finally washed with 1M NaCl solution. Three replicates were made of each plate and the experiment was repeated twice. The NaCl solution elutes the dye in the clearing zone where the cellulose has been degraded into simple sugars by the enzymatic activity.

2.6.4 Protease assay:

The primary screening for extracellular protease production was done by plate assay using skimmed milk agar plates (Abdel Galil, 1992).

**Skimmed milk agar (g/L):**

Skim milk powder 28g
Yeast extracts                          2.5g  
Dextrose                               1g   
Casein                                  5g  
Agar                                    15g

The skimmed milk Agar plates were inoculated with the bacterial culture (P. fluorescens MTCC 9768) and incubated at 37°C for 48 hr. Three replicates were made of each plate and the experiment was repeated twice. The zone of hydrolysis indicates the protease activity of bacteria.

**2.6.5 Chitinase assay:**

The screening of chitinase production in bacteria was done by using colloidal chitin agar (Hsu and Lockwood, 1975).

The colloidal chitin is prepared from crab shells chitin (Sigma). 10g of chitin is dissolved in 400 ml conc. HCl at 4°C. The mixture is then stirred continuously in water bath at 37°C for 15-20 min. 4L of double distilled water was added to the mixture and pH of the suspension was adjusted to 7.0 by addition of 5 N NaOH at 4°C. The supernatant was decanted and the precipitate was collected and washed twice with distilled water. For use, water was added to this precipitate and autoclaved for 150 min at 15lbs.
Colloidal chitin agar (g/L) pH 7.20±.2:

Sodium phosphate dibasic 6g
Potassium dihydrogen phosphate 3g
Ammonium chloride 1g
Sodium chloride 0.5g
Yeast extract 0.05g
Colloidal chitin 10 g
Agar 15 g

The bacterium (*P. fluorescens* MTCC 9768) was inoculated in colloidal chitin agar and was sealed with parafilm. The plates were incubated at 30°C for 5 days. The colloidal chitin hydrolysis was observed by production of clear zone around bacteria.

2.6.6 β-1, 4 glucanase assay:

A modified agar plate assay was used to assay endo-β-1,4-D-glucanase activity (Teather and wood, 1982). The test plates were prepared using 1% CMC and 3% agar mixed with 0.1 M Na-acetate buffer, pH 5.3 and inoculated with the bacteria (*P. fluorescens* MTCC 9768). The plates were sealed and incubated at 37°C
overnight. After incubation, the plates were stained with 1% Congo red (Sigma) for 10-15 min and de-stained with 1.0 M NaCl solution for 15-20 min for several times. Three replicates were made of each plate and the experiment was repeated twice.

2.7 Growth-promoting analysis of antagonistic bacteria:

The antagonist bacteria *P. fluorescens* MTCC 9768 was also tested for its plant growth promoting activity by solubilization of minerals like phosphorous, production of siderophores that solubilize and sequester iron, promotion of plant growth regulators (IAA) and ammonia production.

2.7.1 Phosphate solubilization:

Phosphatase activity of the antagonist bacteria was determined according to Ponmurugan and Gopi, 2006 for its ability to solubilize calcium phosphate present in the Pikovaskaya’s medium (Pikovskaya *et. al.*, 1948).

**Pikovskaya’s agar (g/L) pH 7.3±0.2:**

<p>| | |</p>
<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Glucose</td>
<td>10g</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>5g</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>0.5g</td>
</tr>
</tbody>
</table>
Sodium chloride                                0.2g

Magnesium sulphate heptahydrate   0.1g

The bacterial culture of *P. fluorescens* MTCC 9768 was inoculated on Pikovskaya’s with (tri-calcium phosphate) TCP plates and incubated at 30°C for 2-3 days. Three replicates were made of each plate and the experiment was repeated twice.

**2.7.2 Ammonia production:**

Production of Ammonia by the antagonist bacteria *P. fluorescens* MTCC 9768 was detected according to Cappuccino and Sherman, 1992 and Ahmad *et al.*, 2008. The bacterial culture was grown in culture tubes containing 10 ml peptone water.

**Peptone water (g/L):**

Peptone                                       10g

Sodium chloride                          5g

These tubes were incubated at 30°C for 4 days. After incubation, 1 ml Nessler’s Reagent (Sigma) was added to each tube to determine the production of ammonia indicated by change of color from brown to yellow.
2.7.3 Estimation of IAA:

The production of Indole-aceti-acid (IAA) by the bacteria *P.fluorescens* MTCC 9768 involves conversion of tryptophan into indole-3-acetic aldehyde, as an alternative IAA production pathway. The production of IAA was estimated in presence and absence of tryptophan in growth medium.

Quantitative estimation of IAA was performed using the method of Loper and Scroth, 1986 taking tryptophan (1mg/ml) as standard.

The growth medium for the production of IAA was Yeast Extract Peptone-Dextrose (YPD) medium with and without tryptophan (1mg/ml). The bacterial culture was inoculated in the tubes containing growth medium with and without tryptophan separately. The tubes were incubated at 28°C±2 for 5-6 days. After incubation, the cultures were centrifuged at 3000 rpm for 30 min, and the supernatant was collected. 2 ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Solawaski’s reagent (for 50 ml: 35% perchloric acid; 1 ml 0.5 FeCl$_3$). The tubes were kept in dark for 10-15 min. The development of a pink colour indicates IAA production. Absorbance was taken at 530 nm using UV-visible spectrophotometer (*Pharmacia Biotech Ultrospec 4000 UV/Visible Spectrophotometer*, Britain). The level of IAA produced was estimated by a standard IAA graph. All data are represented as mean and standard errors.
(mean±S.E.). For the IAA production, the variables are analyzed using two-way analysis of variance (ANOVA).

2.7.3.1 Extraction and Identification of IAA:

The bacterial culture of *P. fluorescens* MTCC 9768 was inoculated in 100 ml of nutrient broth amended with 5 mg/ml of tryptophan and incubated at 28°C± 2 for 5-6 days in Bacterial shaker incubator. The bacterial culture was centrifuged at 10,000 rpm for 30 min and the supernatant was collected and acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotator evaporator at 40°C. The extract was dissolved in 100 ml of methanol and stored at −20°C. This fraction (20 µl) was placed on TLC plates (Silica gel HF 254, Loba chemei) with standard IAA (10mg/100ml) and developed in ethyl acetate: chloroform: formic acid (55:35:10). Spots with *Rf* values were visualized by spraying the plates with Salkowski’s reagent (Kuang-Ren *et al.*, 2003).

2.8 Extraction of Secondary metabolites from bacteria:

Extraction of the crude secondary metabolites was done by modified method (Tripathi M, Johri B. N., 2002). The bacterial culture of *P. fluorescens* MTCC 9768 was grown in King’s medium B at 25°C for 72 hr in bacterial shaker incubator at 120 rpm. After incubation, the growth culture medium was centrifuged
at 10,000 rpm for 15 min to obtain the cell free medium. The supernatant of the bacterial culture was extracted so as to obtain crude secondary metabolites. The crude metabolites were extracted by partitioning cell-free broth with organic solvents *viz.* Ethyl acetate, Petroleum Ether, Chloroform, Acetone, Hexane and Methanol in 1:1 ratio in separate flasks. These flasks were incubated at 25°C for 2-3 days at 120 rpm. After 2-3 days, the antifungal compounds were extracted from cell-free broth with organic solvents and evaporated in a rotary evaporator at 45°C to ensure complete solvent removal. The residues thus obtained were dissolved in a minimum quantity of solvents for further studies.

**2.8.1 In vitro antifungal assay:**

The *in vitro* antifungal assay of extracted crude metabolites was done for their efficacy against pathogenic fungi by poisoned food technique (Nene and Thapliyal, 1971). The two volumes (1ml, 2ml) of crude secondary metabolites were incorporated in each well formed on King’s medium B agar plates with sterile cork borer. The agar disc of 4 mm size in diameter was taken from the *T. indica* stock culture and transferred on the center of test plates. The plates containing solvents (without extracts) in the well and fungal agar plug in centre of plates were considered as control. The plates were incubated at 25°C±1 in BOD incubator for 7 days. Three replicates were made of each plate and the experiment was repeated.
twice. After incubation, percentage of inhibition in growth was calculated by using the following formula (McKinney, 1923):

\[
Percentage\ of\ Inhibition = \frac{C - T}{C} \times 100
\]

Where; C= Radial growth of fungus in control plates (mm) and T= radius of zone of inhibition on the plate inoculated with crude secondary metabolites (mm).

All data are represented as mean and standard errors (mean±S.E.). For the percentage of inhibition, the variables were analyzed using two-way analysis of variance (ANOVA).

2.8.2 Identification of antifungal compound:

Thin layer chromatography was carried out with the extracted crude secondary metabolite on TLC plates (Silica gel HF 254, Loba chemei) with acetonitrile: methanol: water (1:1:1) solvent system (Rosales et. al., 1995) and benzene: acetic acid (95:5) solvent system. The crude extract (25 µl) was spotted on TLC plates and the solvent fronts were allowed to run up to 16 cm. Plates were then dried and observed under UV (254 nm and 365 nm) to identify spots.
2.9 Statistical Analysis:

All data are represented as mean and standard errors (mean±S.E.). For the above results, the variables were analyzed using two-way and one-way analysis of variance (ANOVA) to determine differences amongst groups, volume and time period. For all statistical values significance level p<0.05, p<0.01 and p<0.001 were used. The calculations were done with the help of Microsoft Excel and Statistica-10 software.

2.10 In vivo experimentation:

In vivo experiment was conducted in Centre of Biotechnology, University of Allahabad. The climatic conditions were appropriate for the seeding of wheat. The seeding was done in month of October with optimum temperature of (25-28°C).

2.10.1 Preparation of fungal inoculums:

The inoculums of *T. indica* were prepared from old culture grown on PDA at 21°C±4. The conidia were harvested by scraping and was transferred to sterilized distilled water and filtered through nylon mesh. Spore suspensions of *T. indica* were adjusted to $2.1 \times 10^6$ spores/ml with sterile distilled water using a haemocytometer (Derckel et al., 1999).
2.10.2 Preparation of bacteria inoculums:

The bacterial culture of *P. fluorescens* MTCC 9768 was grown for 48 hr in King’s medium B broth at 25°C in bacterial shaker incubator, and then cells were harvested by centrifugation at 5000 rpm for 10 min at 4°C. The bacterial cells were re-suspended in sterile distilled water and the concentration was adjusted to $10^7$-$10^8$ cells/ml (El-Mougy and Abdel-Kader, 2008).

2.10.3 Field experimentation:

The field experimentation was done in a rectangular area of field. The soil was infested with 100 ml of *T.indica* spore suspension ($2.1 \times 10^6$ spores/ml) by drenching. The wheat seeds of two varieties were sown, HD 29 (Resistant variety) and WH 542 (Susceptible variety). The rows were divided as: Control with resistant and susceptible variety of wheat without fungal spores and bacterial inoculums. Tests with resistant and susceptible variety of wheat in presence of fungal spores and bacterial inoculum treatment.

After 20-30 days, when the growth of wheat plants were in tillering stage (usually when plant has 4-5 leaves), the bacterial inoculums (500 µl ) was injected at the crown of the stem. The Disease Severity % (DS) was recorded at the 50th and 70th day after plantation of wheat in in vivo condition by using the scale of Bernier *et al.* (1993) as follows:
\[ \text{Disease severity (DS \%) = } \frac{n \times v}{M \times N} \]

Where, \( n \) = Number of plants in each category \( (v) \) = Numerical value of symptoms category \( (N) \) = Total number of plants \( (M) \) = Maximum numerical value of symptom category.

### 2.11 Identification of antifungal compound:

The identification of the antifungal compound from *P. fluorescens* MTCC 9768 was performed by FTIR, HPLC and LC-MS.

#### 2.11.1 Fourier Transformation Infrared Spectroscopy (FTIR):

The FTIR analysis was performed to investigate the presence of functional groups in the antifungal compound extracted secondary metabolites from *P. fluorescens* MTCC 9768. The FTIR analysis was done on Thermo Scientific Nicolet iS50FT-IR in the mid IR region of 400-4000 cm\(^{-1}\) with 16 scan speeds. The sample was prepared using pure KBr (5:95). The pellets were fixed in the sample holder and analyzed.

#### 2.11.2 High Performance Liquid Chromatography (HPLC):

The antifungal compound extracted from the secondary metabolites from *P. fluorescens* MTCC 9768 was monitored by analytical HPLC (Waters e2695 coupled with 2998 Photo diode array detector) by gradient mode of elution for
investigating the UV absorbance (spectra) and purity. The analytical data were evaluated using Empower 3 software.

The separation was achieved by using YMC-Propak – C$_{18}$-250 mm*4.6 mm* 3µm column at 25°C with 1ml/min flow rate. The two mobile phases were used in gradient mode of elution, Mobile phase A (50 mM ammonium dihydrogen phosphate in milli-Q water, pH 3.0 with diluted orthophosphoric acid) in 1000 ml of milli-Q water and Mobile phase B (acetonitrile: water: terta hydrofurane:: 75: 15: 10 (v/v)), both the mobile phases were sonicated for degassing. The sample was prepared using methanol as diluents at 5mg/ml sample concentration. The monitoring wavelength was 305 nm. The HPLC gradient used for compound is as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A (%)</th>
<th>Mobile Phase B (%)</th>
</tr>
</thead>
<tbody>
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<td>0</td>
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</table>
2.11.3 Liquid chromatography-mass spectroscopy (LC-MS):

The antifungal compound extracted from *P. fluorescens* MTCC 9768 was subjected to (LC-MS) analysis for molecular weight determination. The MS analysis was performed in LC-Agilent 1200 with 6330 ion trap LC-MS. The sample was ionized in electron spray with positive polarity. The electron spray ionization was operated at 4.5 kV and the loop capillary temperature at 300°C. The gradient mode of elution was used for molecular weight determination. The two mobile phases were- Mobile phase A (0.2% triethylamine, 2.72 g of potassium dihydrogen phosphate (pH 6.0 with orthophosphoric acid) in 1000 ml milli-Q water, 5ml of acetonitrile was added and sonicated for degassing, Mobile phase B was taken as acetonitrile. The sample was injected at 10μl with a flow rate 0.5 ml/min in LC column (Poroshell EC - C18 – 150 mm* 4.6 mm* 2.7μm) at 30°C at 255 nm. The gradient conditions are as follows:

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Mobile Phase A (%)</th>
<th>Mobile Phase B (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>7</td>
<td>90</td>
<td>10</td>
</tr>
<tr>
<td>12</td>
<td>70</td>
<td>30</td>
</tr>
<tr>
<td>15</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>19</td>
<td>25</td>
<td>75</td>
</tr>
<tr>
<td>20</td>
<td>97</td>
<td>3</td>
</tr>
<tr>
<td>27</td>
<td>97</td>
<td>3</td>
</tr>
</tbody>
</table>
2.12 Designing of Biosensor:

The work on development of Biosensor against *T. indica* was accepted and approved by Indian Institute of Technology (IIT) Bombay under Indian Nanoelectronics User Program (INUP).

2.12.1 Production of antibodies against antigen:

The anti- teliospore polyclonal antibodies (IgG) was purchased from Pierce Custom Services, Thermo Fisher Scientific.

2.12.2 Immobilization of antibodies on cantilever:

The silicon substrate cantilever was purchased from Nanosniff Technologies, Mumbai.

I. Cleaning of Cantilever:

Before the functionalization, the cantilever was cleaned to remove contaminations from the silicon surfaces in Piranha solution (97% H$_2$SO$_4$ in 30% H$_2$O$_2$ at ratio of 1:1) for 20 min and rinsed twice with sterile-filtrated water and once with deionized water. The cleaning procedure was performed twice. Thereafter TiO$_2$/Au layers were deposited by e-beam deposition and the interface activated with proteins (Arntz *et al.*, 2003).
II. Instrumentation:

The frequency response of the micro cantilever during excitation was measured using a Multi Mode™ AFM (Digital Instruments, Santa Barbara, CA). The light of the laser diode was focused on the apex of the cantilever, from where it was reflected onto a position-sensitive detector. Thereafter, the signal was transmitted to a frequency generator and frequency analyzer.

III. Cantilever Functionalization:

The immobilization of *T. indica* spores was performed via anti-teliospore polyclonal antibodies (IgG) attached in tilting manner on protein-A activated silicon cantilever. The protein-A coating was performed directly on gold-coated cantilevers. IgG and protein-A were adjusted to 100 µg/ml in 0.15 M PBS (pH 7.4). The silicon cantilever was silanized using a 1% (3-aminopropyl)-triethoxysilane solution in toluene for 1 hr at 24°C. The amino-groups of the final silanized layer were activated by 2.5% glutaraldehyde (in PBS buffer) for 1 hr at 24°C and then washed in PBS for three times. Thereafter, the pre-activated cantilever was incubated in protein-A solution over night at 4°C and washed in PBS. To functionalize the surface with IgG, the cantilever was placed in a capillary device containing IgG solution for 2 hr at room temperature. After incubation, the cantilever was washed in PBS.
IV. Detection of spores:

To prepare the final spore suspension for spore immobilization, old *T. indica* disc bearing spores was diluted with 10 ml sterile 0.15 M PBS (pH 7.4), mixed for 5 min and filtered through nitrocellulose filters to separate spores from mycelia. The antibody-functionalized cantilever was incubated in quartz micro capillaries. The sensor cantilever was exposed to *T. indica* spores suspension in PBS and control cantilevers were immersed in PBS without *T. indica* spores simultaneously. After incubation for 45 min, microarrays were first rinsed in PBS buffer and then in a nutritive solution (PBS/potato/dextrose extracts mixture in ratio of 1:1). Potato/dextrose extract was used as a soluble nutritive source for fungal growth. Afterward, the functionalized cantilevers were mounted into the MultiMode device for the measurement.