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4. Isolation, Identification of Fungi and Assessment of Disease

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4.1 Introduction:

It is the basic work for any plant pathological research. It is done some common steps which are discussed in this chapter.

4.2 Collection of Rhizospheric Soil and Diseased Sample:

Rhizospheric soil adhere to root of plant was collected in separate sterile polythene bags and labelled properly.

For the diseased sample, infected parts of maize were collected in sterile polyethylene bags and labelled properly. Both the rhizospheric soil and infected parts were brought to the Laboratory for the isolation.

4.3 Isolation of Mycoflora from Soil Sample:

Isolation of mycoflora was conducted from rhizospheric soil of plant through serial dilution method as demonstrated by Johnson *et al*., 1972 (**Figure 4.1**).

In this method one gram soil was measured, dissolved in 10ml of sterile distilled water aseptically in 250ml of Erlenmeyer flasks and serially diluted in the range of 10^{-3} to 10^{-5} with sterilized distilled water.

Fifteen millilitres of Potato dextrose agar (PDA) media amended with antibiotic (to allow of fungi only) was poured aseptically onto petriplates and allowed for solidification.

Then 1.0ml of soil suspension from 10^{-3} dilution were added to the petriplates/petridish and spread uniformly, sealed with parafilm and incubated at 28^0C for 7 days.

Individual colony being appeared after next few days of incubation were picked up from the petriplate and transferred on freshly prepared PDA slants or PDA plates as pure culture and incubated at 28 ± 2^0C for 7 days and then stored at -4^0C for further use.

Total number of similar and dissimilar colonies i.e. colony forming units (c. f. u.) appeared in the petriplates may be counted.

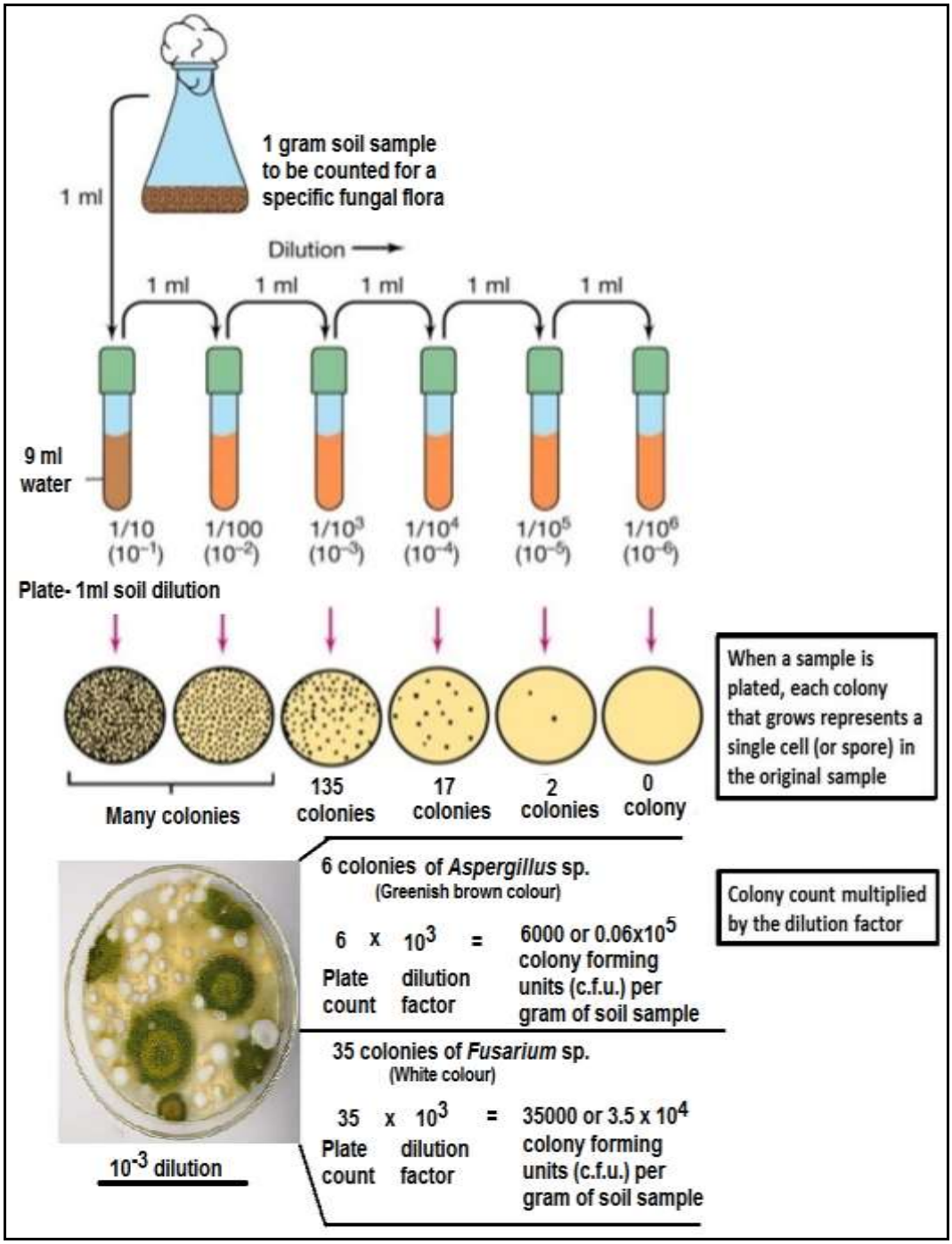

Figure 4.1: Serial dilution method for counting colony forming units (c. f. u.). Serial dilution was prepared by adding one gram of soil sample to 9ml of sterile distilled water (SDW) in a sterilized flask, shaken well, a serial dilution (10^{-1} to 10^{-6}) were made in the same method. One ml of each dilution was poured in each Petri dish (9cm in diameter) containing prepared medium (PDA + antibiotics) by sterile pipette each sample made by three replication plates and incubated at 25°C for 7days for appearing fungal colonies.

4.4 Koch's Postulates:

Koch's postulates are four criteria designed to establish a causal relationship between a causative microbe and a disease of plant. The postulates were formulated by Robert Koch and Friedrich Loeffler in 1884 and refined and published by Koch in 1890.

Based on his experiences, Koch set out the four steps or criteria that must be satisfied before a microorganism isolated from a diseased human, animal, or plant can be considered as the cause of the disease.

These four steps, rules, or criteria are known as "Koch's postulates." Here it has been generalized to plant diseases with modification (**Figure 2**). The four steps are-

A. Step 1:

The suspected microbial causal agent must be found in abundance in all plants suffering from the disease, but should not be found in the healthy plant. Diseased sign and symptoms in plants should be noted.

B. Step 2:

The suspected microbial causal agent must be isolated from a diseased plant and grown in pure culture. Microbial growth pattern i.e. colony morphology on PDA plates and microbial morphology under microscope should be noted. It is done as follows.

The disease part was cut into different segments, washed with distilled water to remove debris and treated with 0.1% mercuric chloride for 2-3 min and rinsed with distilled water. Then it was treated with 70% alcohol for one minute and washed four times with sterile distilled water.

Treated segments were air dried aseptically and transferred onto PDA plates and incubated at 28 ± 2^0C until desired growth pattern on colony of microorganism was obtained.

Colony morphology and microbial morphology was noted and transferred onto freshly prepared PDA slants in pure culture.

C. Step 3:

The pure culture of microorganism then used to inoculate healthy plant of same cultivar. It should cause disease with sign and symptoms similar to step 1, when introduced into a healthy plant of identical cultivar.

D. Step 4:

The microorganism must be re-isolated on PDA plate from the inoculated, diseased experimental host plant following the step of step 2. Colony morphology and microbial morphology of the recovered agent must have the same characteristics as in step 2.

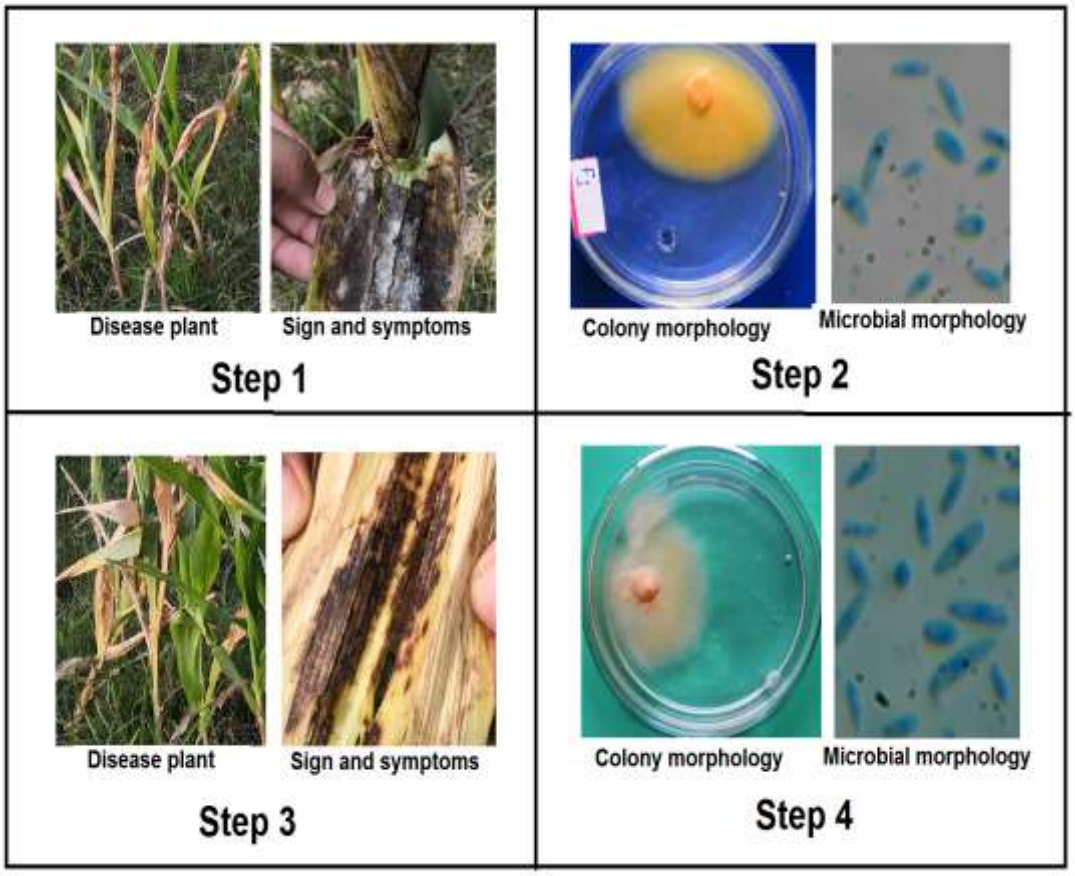

Figure 4.2: Koch's Postulates- Four Steps, Rules, or Criteria.

Step 1: the suspected microbial causal agent must be found in abundance in all plants suffering from the disease, and diseased sign and symptoms in plants should be noted.

Step 2: The suspected microbial causal agent must be isolated from a diseased plant, grown in pure culture and microbial growth pattern i.e. colony morphology on PDA plates and microbial morphology under microscope should be noted.

Step 3: the pure culture of microorganism when introduced into a healthy plant of identical cultivar, it shown sign and symptoms similar to step 1.

Step 4: the microorganism re-isolated on PDA plate from the inoculated, diseased experimental host plant and it must have the same characteristics as in step 2.

4.5 Identification of Mycoflora:

Mycoflora were identified on the basis of morphological characteristics of the colony, conidia spore, colony features (**Figure 4.3**) with the help of standard texts (books) used for identification of mycoflora depending on their taxonomic keys. Further culture was confirmed by Indian Type Culture Collection with ITCC number.

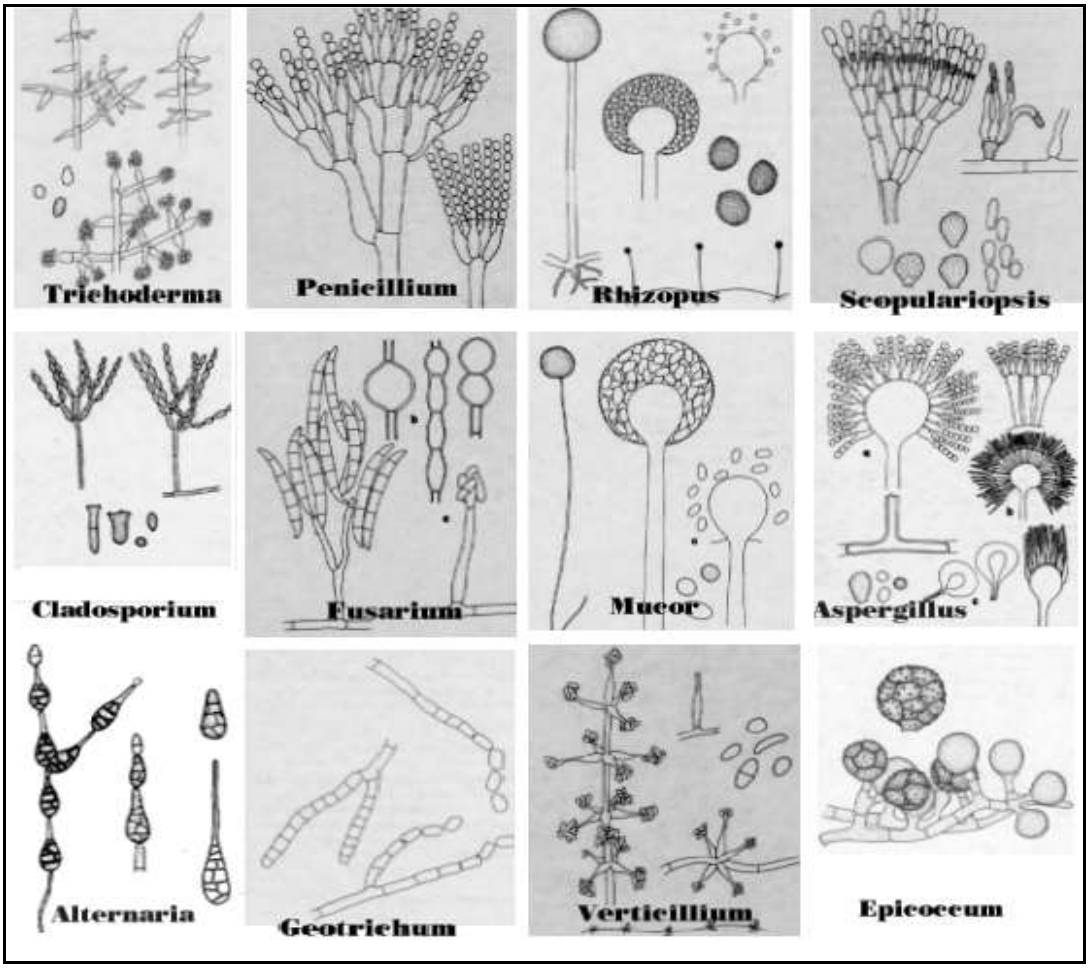

Figure 4.3: Some Common Genera of Mycoflora

4.6 Molecular Identification:

Fungi are morphologically, ecologically and metabolically diverse. By using morphological features alone in the identification of fungi to the species level is difficult.

It is unable to solve the confusion of anamorphs (asexual reproductive stage) and telomorphs (sexual reproductive stage) of fungi.

Now molecular identification is considered for species level identification of fungus which was done through the three nuclear ribosomal genes and ITS region which is the official DNA barcoding marker for species-level identification of fungi using NCBI-BLAST search for DNA barcoding with numerous curated molecular databases containing fungal sequences to augment or supplant ITS in species-level identification of fungal groups and construct phylogenetic trees from DNA sequences to facilitate fungal species identification.

It is further recommended that, whenever possible, both morphology and molecular data may be used for fungal identification.

4.7 Preparation of Fungal Inoculum:

Spore Suspension:

A. Colorimetric Method:

Fungal pure culture was multiplied on 250ml Erlenmeyer flask containing 150ml Potato dextrose broth (PDB) at 23 ± 2^0C for 7 days. After 7 day of incubation, culture broth was centrifuged at 10,000 rpm for 10 min. The pellets were suspended in sterile distilled water and wash repeatedly for four times. The washed fungal pellet was made into a turbid solution with sterile distilled water. The OD of the solution was adjusted to 0.45 at 610 nm at colorimeter to obtain $1x10^8$ cfu ml^{-1} as described by Sudisha *et al*., 2006 (Sudisha, Niranjana, Umesha, Prakash, & Shetty, 2006).

B. Hemacytometric Method:

It is an excellent method for determining numbers sporangia, zoospores or spore of fungi. It was done as follow. Take hemocytometer and coverglass, then clean and dry with 70% ethanol and Kimwipes. Dampen the mounts with a wet kimwipe or by exhaling on the hemocytometer. Place the hemocytometer coverglass across the mounts, center, and gently press down. *Hemocytometer coverglass is thicker than standard coverglass and does not warp under liquid surface tension.* Insure that the spore suspension is well-mixed and pipette 10 µl drop of spore suspension into one inlet of the hemocytometer. Wait for 1 minute for cells to settle on the grid. Place the hemocytometer under microscope and find the grid. Each corner square has an area of 1 mm^2 and a depth of 0.1 mm for a total volume of $1x10^{-4}$ ml. Count the total cells in all 4 corner-squares of the grid **(<u>Figure 4.4</u>)**. In *practice, cells may be distinguished from debris. Continuously shift the focal plane up and down while counting to look for rings around cells.* After counting the cells in all 4 corner-squares, divide by 4 to get the average number of cells per 1 mm^2 cell area. Multiply this number by $1x10^4$ to obtain the average number of cells per ml. *For cells that lie on an edge, only count cells that cross the top or left edge of a corner square.* Disassemble the hemocytometer. Clean and dry the hemocytometer and coverglass with 70% ethanol, and store them in a safe location.

Example: If the calculated average number of spores per 1 mm^2 corner squares of the hemacytometer is 60. Then number of spore per millilitre (spore/ml) = $60x10^{+4}$ spores /ml = $6x10^{+5}$ spore/ml = 600,000 spores/ml.

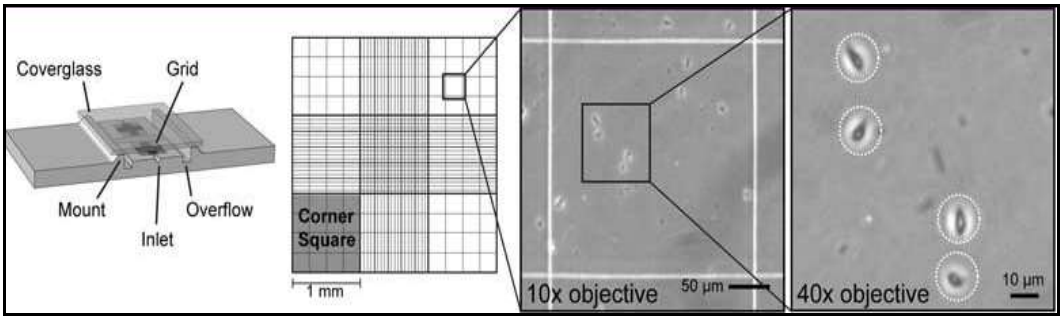

Figure 4.4: Hemocytometer used for counting spore.

4.8 Pathogenicity Test:

The pathogenicity test is the main criterion for identification of fungus suspected of being the causal agents of a plant disease.

This involves reproduction of lesions following artificial infection of susceptible hosts under greenhouse conditions.

Occasionally, pathogenicity tests may be performed under controlled laboratory conditions. The choosing of resistant variety of crops against a fungal pathogen is one of the most important ways for management of plant disease.

From large varieties of a particular crop plant, resistant variety was chosen following pathogenicity test of a fungus causing severe disease in the said plant.

It is done by leaf inoculation technique and root inoculation technique.

4.8.1 Leaf Inoculation Technique:

Pathogenecity of test pathogen was performed following the whole plant inoculation method of Dickens and Cook (1989). For this nursery seedlings (raised through seed germination) of 6 month old plants of different varieties were inoculated with the test pathogen.

Inoculation was done by spraying conidial suspensions ($1x10^6$ conidia/ml) prepared from 10 days old cultures of test pathogen grown on appropriate medium (potato dextrose agar slants) In control set plant were sprayed with sterile distilled water. The plants were kept for 48 hours in glass chamber to maintain high humidity.

A. Leaf Disease Assessment:

The number of lesions developed on the leaves after 4, 8, 12 and 16 days of inoculation were counted and diameters of each lesion were measured.

The results were computed following the method of Sinha and Das (1972). The diameters were categorized into four groups and a value was assigned to each group as follows:

- Very small-restricted lesions of 1-2 mm diameter: 0.1
- Lesions with sharply defined margins of 2-4 mm diameter: 0.25
- Slow spreading lesions of 4-6 mm diameter: 0.5
- Spreading lesions of variable size (beyond 6 mm in diameter) with diffused margin: 1.0

The number of lesions in each group was multiplied by the value assigned to it and the sum total of such values was noted and disease index was computed as the mean of observations on fifteen plants per treatment.

Results (mean foliar disease index) were calculated and presented (**Table 4.1**).

Table 4.1: Pathogenicity of test pathogen of different varieties (based on mean foliar disease index)

Variety	Days after inoculation			
	4 days	8 days	12 days	16 days
	Mean foliar disease index/plant*	Mean foliar disease index/plant	Mean foliar disease index/plant	Mean foliar disease index/plant
A	1.54	5.54	9.64	12.42
B	1.28	3.61	6.01	10.20
C	1.00	2.92	6.34	9.80
D	0.85	2.28	5.10	5.90

4.8.2 Root Inoculation Technique:

Root disease assessment was done on the basis of visual observation of lesion on roots after 4, 8, 12, 16 days of inoculation as described by Kobriger *et al*. (1984). Different varieties of plants were taken for pathogenicity of test pathogen on roots to determine root disease index.

In the beginning of the experiment sterile seedbeds were prepared. To prepare sterile seed bed a plastic pot (10 cm in diameter) was filled up with 500g soil with adequate water for moisturizing the bed, covered with polythene sheet and autoclaved at 1.5 lb pressure for 30 minutes.

After cooling, polythene cover of the pots was removed. Plant seeds, 5 to 7 in nos. per pot were sown in 2-3 cm depth in the sterile seed bed. The pots were watered regularly and allowed to raise seedlings. Seedlings of the pots were inoculated with conidial suspension ($1x10^6$ conidia/ml) prepared from 10-days old culture of test pathogen grown in sterile potato dextrose agar slants.

A. Root Disease Assessment:

Root diseases were assessed following the method of Kobriger *et al.* (1984). About 50 viable seeds were taken for each varietals test.

Following inoculation seedlings were sampled after 4, 8, 12 and 16 days. The whole experiment was performed in a glass chamber to maintain high humidity.

Severity of the symptoms was graded into five disease classes (**Table 4.2**). The seedlings were uprooted carefully and graded as mentioned bellow.

Mean values of the 15 plants were considered as mean root disease index. Symptoms appeared on root after 4, 8, 12 and 16 days of inoculation with test pathogens which were assessed following root disease assessment procedure (**Table 4.3**). Results (mean root disease index/plant) were calculated and presented (**Table 4.4**).

Table 4.2: Disease classes of root after infection with test pathogen

Disease classes	Feature of lesion
0	Indicates no disease lesion on root
1	Indicates slightly affected brown lesion on roots
2	Indicates deep brown lesion on roots
3	Indicates severe browning of roots turn to blackish
4	Indicates completely damaged roots with black lesions

Table 4.3: Procedure of calculating root disease index after infection with test pathogen

	Plot (P)	Disease classes (DC) after 16 days of inoculation with test pathogen				
	Plot # 1(7 seedlings)	0	1	2	3	4
	Plot # 2(5 seedlings)	0 0 0	2 1 2	2 1 2	2 1 2	1 2 3
	Plot # 3(9 seedlings)	0	5	5	5	6
Total diseased plants (T)	5+5+5+6 = 21					
P x DC		0	5	10	15	24
Σ (P x DC)	0+5+10+15+24 = 54					
Disease Index =		[Σ(P x DC) x 100] / (T x 4)				64.28

Table 4.4: Pathogenicity of test of pathogen on different varieties (based on mean root disease index)

Variety	Inoculation period			
	4 days	8 days	12 days	16 days
	Mean root disease index/plant	Mean root disease index/plant	Mean root disease index/plant	Mean root disease index/plant
A	15.3	23.7	48.2	64.2
B	12.7	15.6	22.5	35.8
C	10.4	16.7	18.8	31.6
D	9.5	15.7	14.6	28.2
E	8.3	9.5	10.8	21.7

4.9 References:

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