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STUDIES ON THE ASSESSMENT OF BIOCHEMICAL MODIFICATION IN CAPSICUM ANNUUM INDUCED BY PATHOGENIC FUNGI

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ABSTRACT

Host-parasite relationship arise from long-term relationships between organisms living in a particular environment. The nature and extent of the relationship determine the type of relationship that exists between organisms living together. The parasite has its determinants of virulence that allow it to invade and damage the host and to resist the defenses of the host. Host-parasite associations generally result in four main associations: parasitism, mutualism, commensalism, and phoresis.

KEYWORDS: Host-parasite, parasitism, mutualism, phoresis.

INTRODUCTION

The Greek word kapsimo, which means "to bite" or "to swallow," is where the term "capsicum" originates. The Solanaceae family and genus Capsicum include chilli peppers (*Capsicum annuum L.*). The names are Mirch in Hindi, Morok in Manipuri, Mulaga in Tamil, Pacha Mulagu in Malayalam, Hmarcha-te and Vai-hmarcha in Mizo, and Kedi-chusi in Angami. The genus includes chili peppers, bell peppers, ajíes, habaneros, jalapeños, ulupicas, and pimientos, well known for their economic importance around the globe.[1] A symbiotic connection is one in which a symbiont spends all or a portion of its existence inside or on top of a living host; typically, the symbiont provides benefits while the host suffers some sort of injury, and it typically has a larger capacity for reproduction than the host.[2] Rather than concentrating on the processes underlying interactions within a host, descriptions of parasite community ecology frequently highlight trends of parasite abundance across host populations.[3] These

proteins that are not or only at basal concentrations detectable in healthy tissues but for which accumulation at the protein level has been demonstrated upon pathological conditions and related situations in at least two or more plant-pathogen combinations is the definition of PR proteins.[4]

MATERIALS AND METHODS

1. Selection of the Host Species

The host species[5] has been selected from the plant nursery. *Capsicum annuum L.* was selected for the experimentation.

Fig. 1.0: Host Plant of *Capsicum annuum*.

2. Collection of infected host Plant parts from open agricultural fields

The infected leaves were selected having symptoms of fungal disease and were isolated from the local crop fields of Bhagalpur, Bihar. The samples were collected during early morning in the month of August 2022. The sampling was done randomly from the different corners and the centre of the crop field. The infected leaves were sealed in a sterilized zipper poly bag and brought to the departmental laboratory of biotechnology, TMBU. The samples were stored at -4°C till further experimentation.

Fig. 2.0: Capsicum leaf showing fungal infection.

3. Isolation of Fungal parasite

Diseased plant tissue was cut from the advanced edges of the lesions into small pieces (5mm diameter) with the help of a sterilized borer in aseptic condition of the Laminar Air Flow Hood. Each ex-plant was transferred on Potato Dextrose Media (PDA) plates (25 ml)[6]. Then the plates were incubated for 72 hours in an inverted position at 27 ±2° C. The fungal colonies grown were purified upon PDA slant for their further identification and pathological studies.

Table 1.0: PDA media composition.

Potato infusion	200 gm
Dextrose	20 gm
Agar	20 gm
Distilled water	1 liter

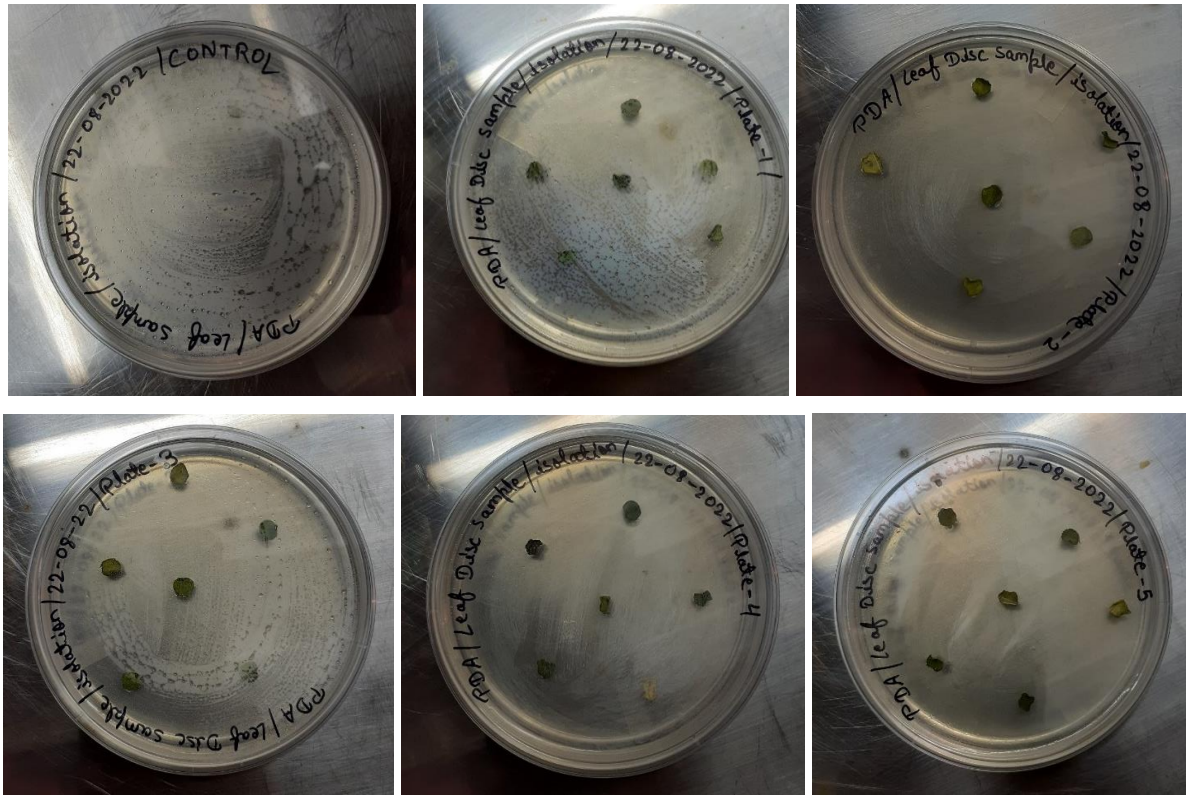


Fig. 3.0: Isolation of fungi on Petri plate.

Fig. 4.0: Slant Preparation of isolation of fungi.

4. Identification of Fungi isolated from ex-plant

The morphological identification and characterization were done by Lactophenol blue (LPCB).[7] The chitin wall of the fungi becomes permeable due to the application of lactophenol that enables the entry of staining dye cotton blue within the cytoplasm of the whole fungal body. To identify the fungi under study, morphological characters were used like hyphae, conidial heads, etc., by using the Gilbert and Barnett method.[8]

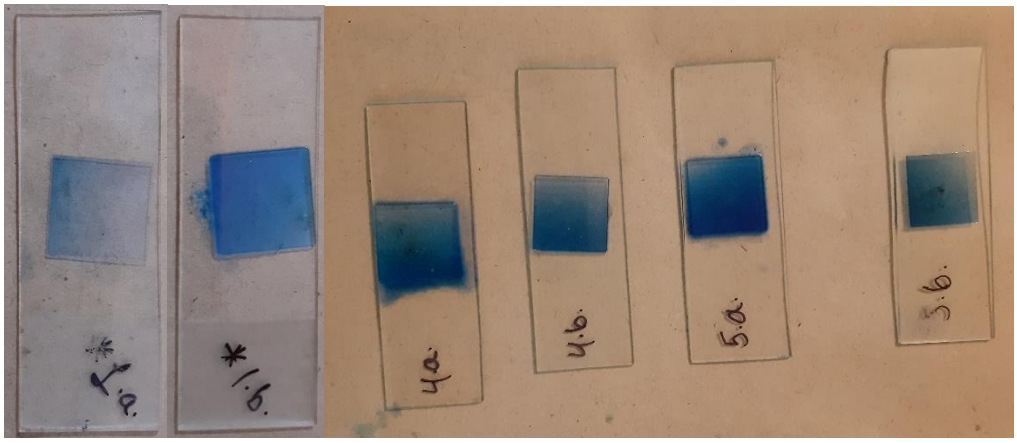


Fig. 5.0: Slides of fungi to view morphology under microscope.

5. Infestation of host plant with isolated fungal pathogen

The different fungal colonies were cultured in suspension of 250 ml conical flask and allowed for sporulation under static condition. From the different fungal colonies spores were isolated to make a suspension of 1 x 10^5 per ml for infestation of fungal pathogens. To obtain an

accurate spore count a homogenous suspension with individual cells was made. 10 µl of spore was added in the solution to each side of the hemocytometer.[9]

Average number of spores in one large square (I) = $\frac{i+ii+iii+iv+v}{5}$

$C_1V_1 = C_2V_2$

C = Final concentration that is needed in ml

V = Final volume that is needed

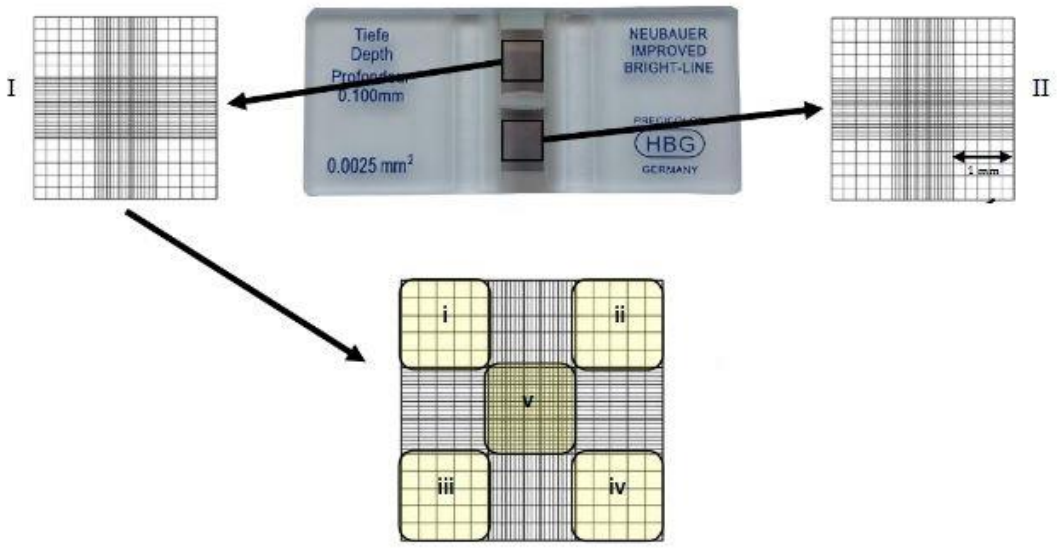


Fig. 6.0: Hemocytometer.

Fig. 7.0: Fungal spores infested in *Capsicum annuum* leaves.

6. Biochemical Screening of metabolite of Control and infested plant

i) Alkaloids by Mayer's method - This test is performed by adding 2 ml of concentrated HCl to 2 ml of the corresponding plant extract samples, then adding a few drops of Mayer's reagent. The presence of alkaloids in the tested sample was verified by the appearance of either green colour or white precipitate.[10]

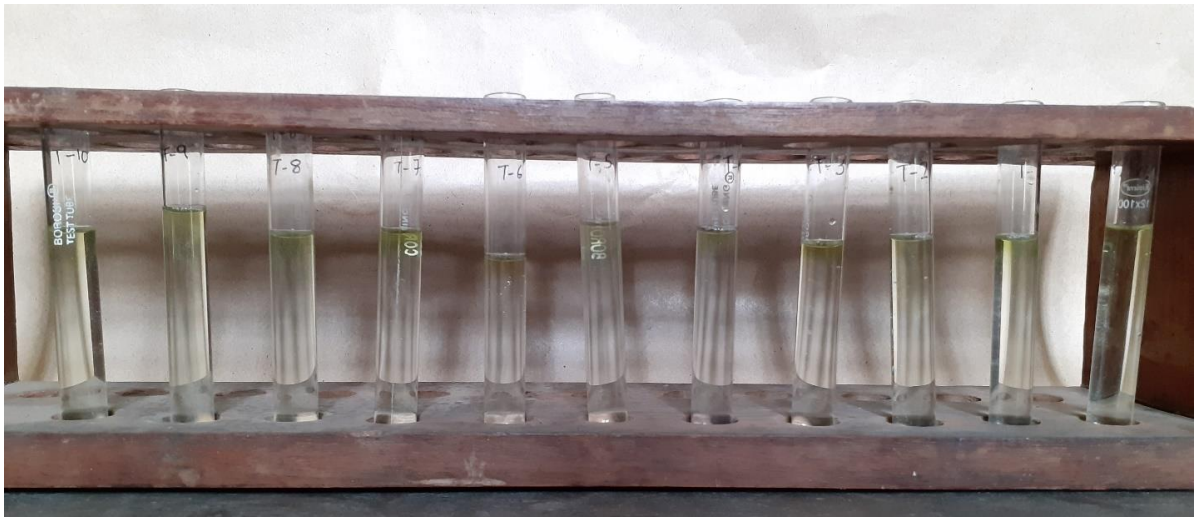


Fig. 8.0: Alkaloid.

ii) **Saponins by Foam Test –** 1 ml solution of extract was diluted with distilled water to 20 ml and shaken in a graduated cylindrical for 15 minutes, the development of stable foam formation shows the presence of saponins.[11]

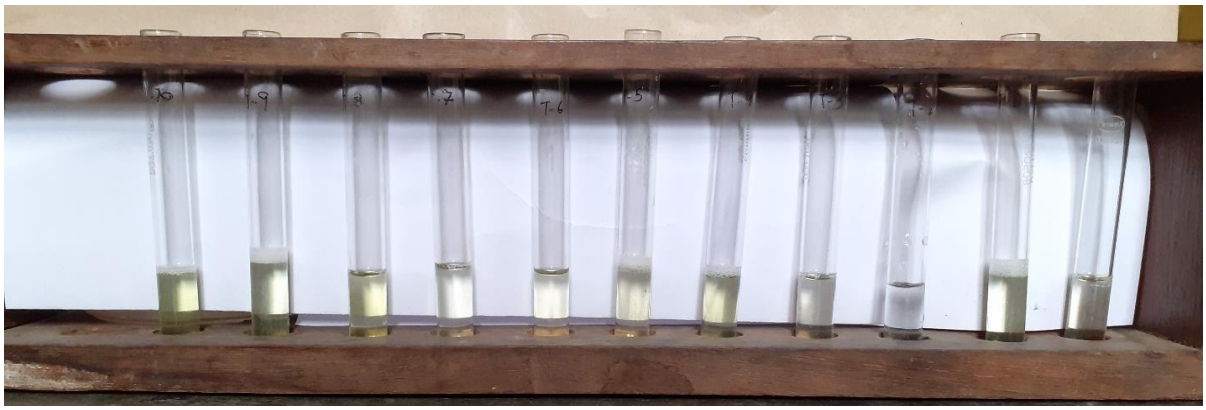


Fig. 9.0: Saponin Test.

7. **Screening of host antiparasitic enzymes**

a. **Cellulase Test (Glycosidase Test by Agar diffusion) -** This biochemical test determines whether an analyte contains an enzyme that breaks down carbohydrates. Glycosidic enzymes are detected via the gel diffusion test using starch. When the extract's glycosidase enzyme breaks down the extract that has diffused through the agar well, a hollow area forms surrounding the well. The addition of I/KI solution, which results in purple coloration in the non-digested starch area of the gel and no coloration at the degraded starch region, confirms the outcome.[12]

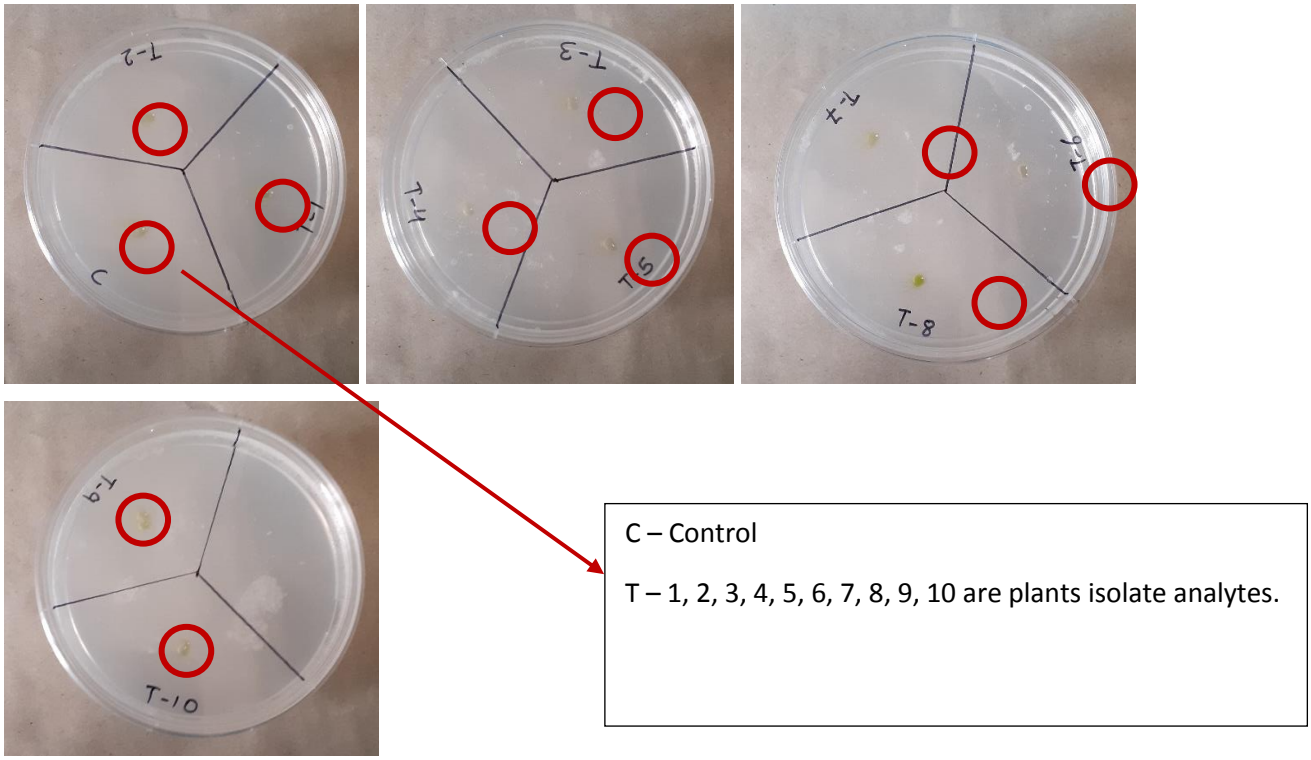


Fig. 10.0: Glycosidase Test of different plants samples before adding IKI Solution.

b. Pectinase Test (Chitosan Test by Agar diffusion) – Chitinase enzyme is a hydrolytic enzyme that break down glycosidic bond in chitin. Chitinase are produced by higher plants to defend themselves against pathogenic attacks by degrading chitin in the cell walls in fungi and bacteria. A chitin agar plate is prepared with the mixture of bromocresol purple (indicator). The appearance of crimson red color suggests the conversion of chitin into chitosan which require the activity of chitinase enzyme. Chitin agar plate has been used for identifying chitinolytic activity in plant and observing clear zone around the well.[13]

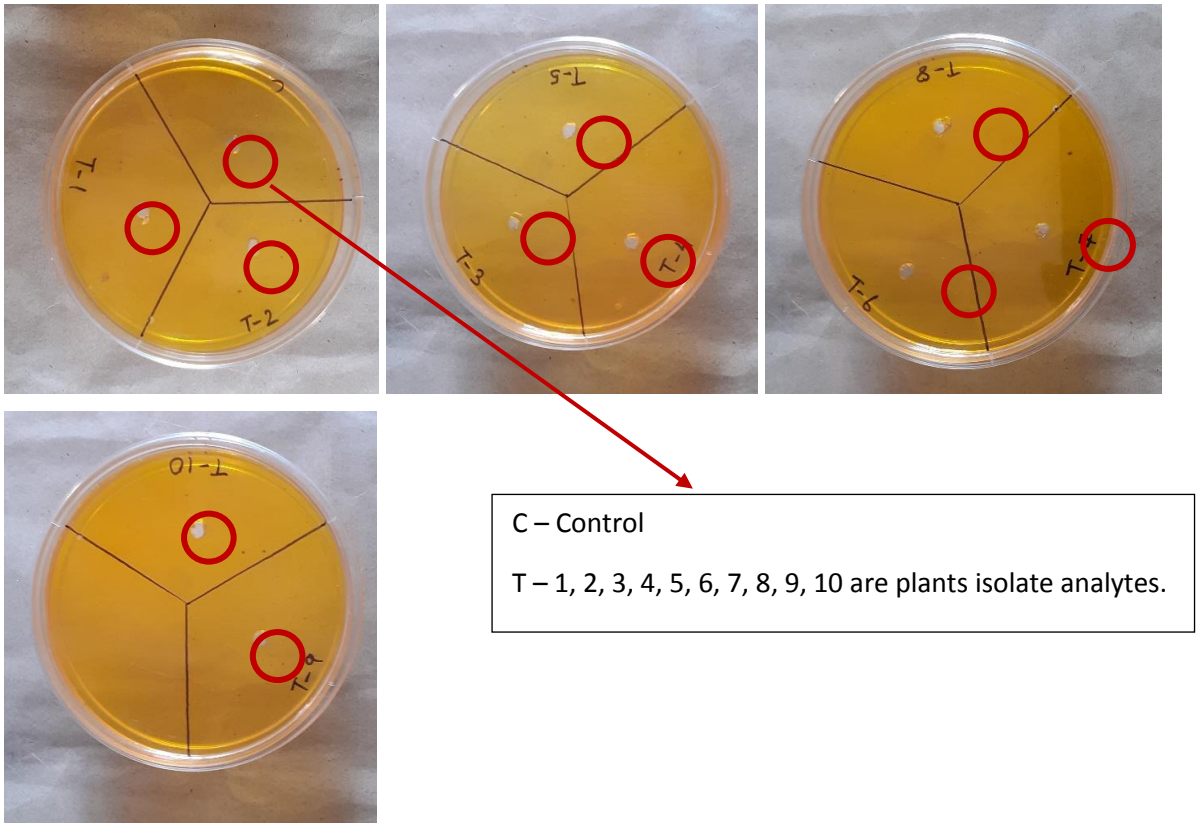


Fig. 11.0: Chitosan Test.

8. Screening of Short Peptide in host tissues

Antimicrobial peptides (AMPs) and pathogenesis-related (PR) proteins are two distinct groups of molecules that are triggered by defence-related signalling molecules and phytopathogens.[14] The infected leaves were plugged from the test plants and homogenized a PBS buffer (pH 7.0) to make tissue extract. The extract was centrifuged (5000 rpm, 10 min) and the supernatant was taken to prepare the sample for electrophoresis. In NSDS-PAGE, 7.5µl of protein sample were added to 2.5µl of 4X NSDS sample buffer (100 mM Tris HCl, 150 mM Tris base, 10% v/v glycerol, 0.0185% w/v Coomassie G-250, 0.00625% w/v Phenol Red, pH 8.5).[15]

RESULT AND DISCUSSION

1. Selection of host species

Capsicum annuum L. has been selected and collected from the plant nursery from Bihar Agriculture University, Sabour.

2. Collection of infected host plant parts

From open agriculture fields of *Capsicum annuum* infected leaves were collected and cut out in form of 5 mm disc for the isolation of fungal pathogens.

Fig. 12.0: Ex-plant of *Capsicum annuum L.*

3. Isolation of fungal parasite from infected plant

Total of 10 fungal isolates were obtained which get purified over PDA slant and morphologically identified for colony characters and mycelial traits.

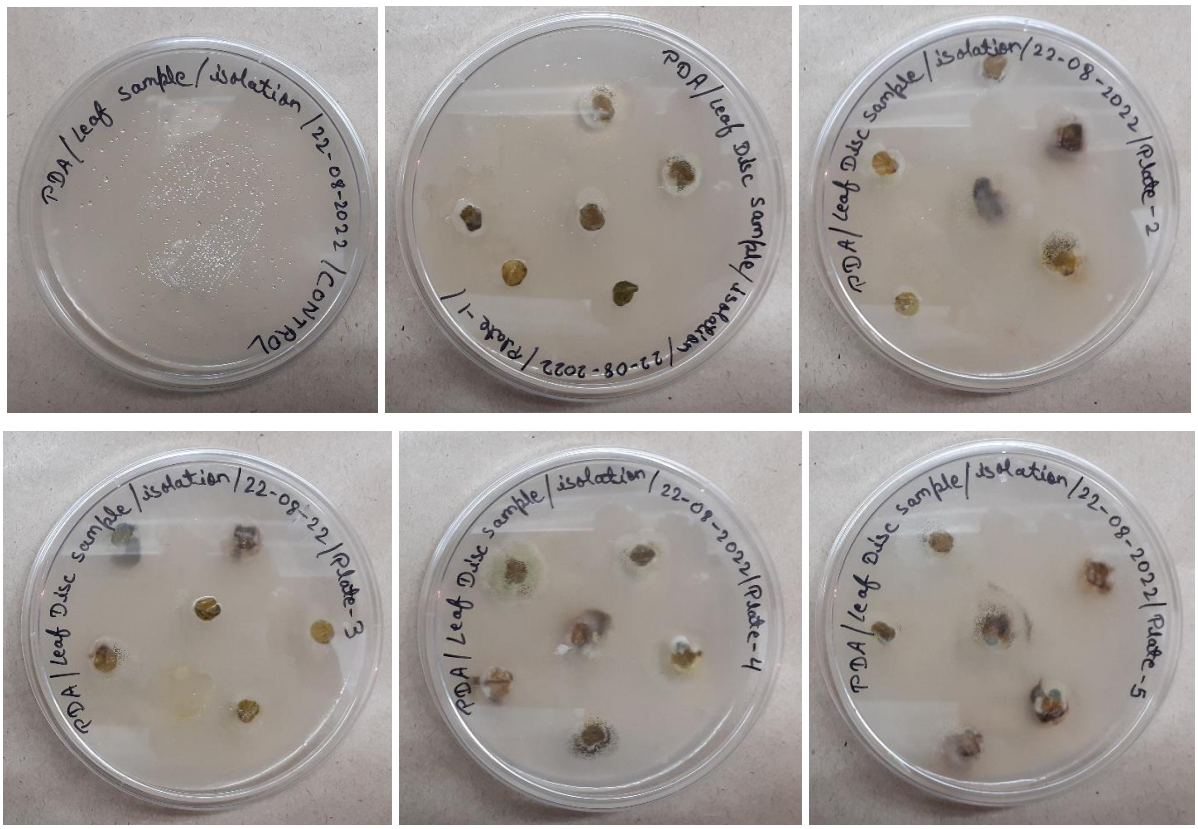


Fig. 13.0: Isolates of fungi grown on Petri Plates.

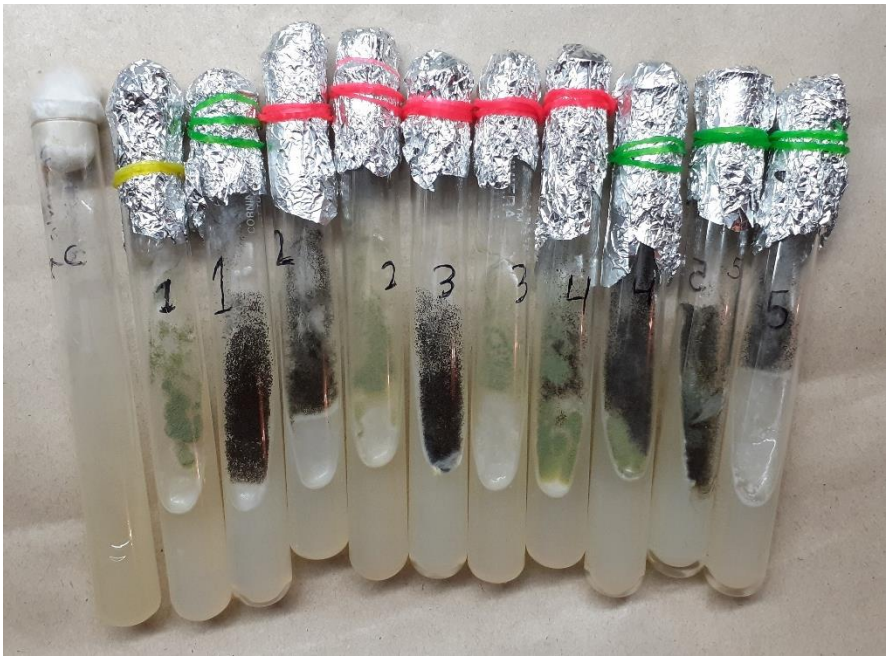
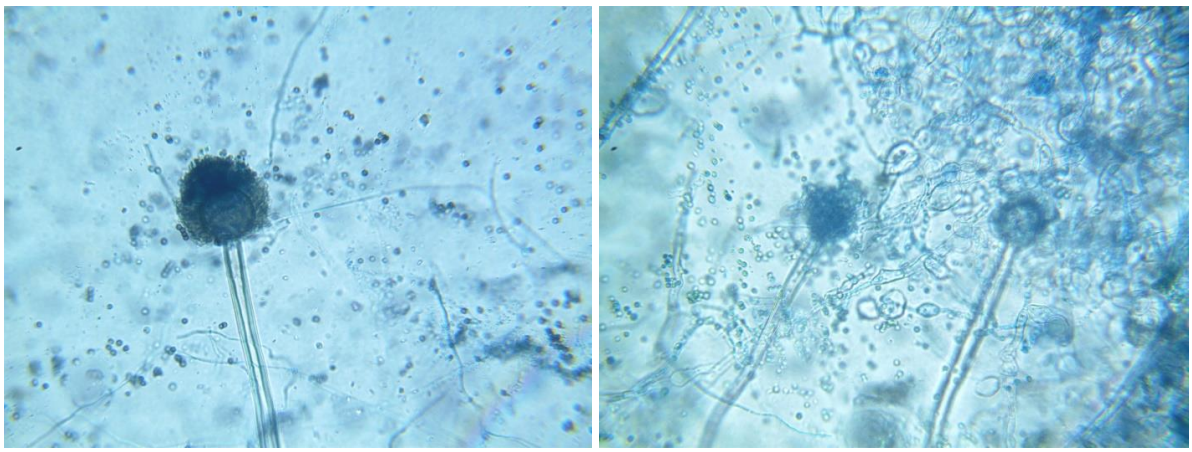


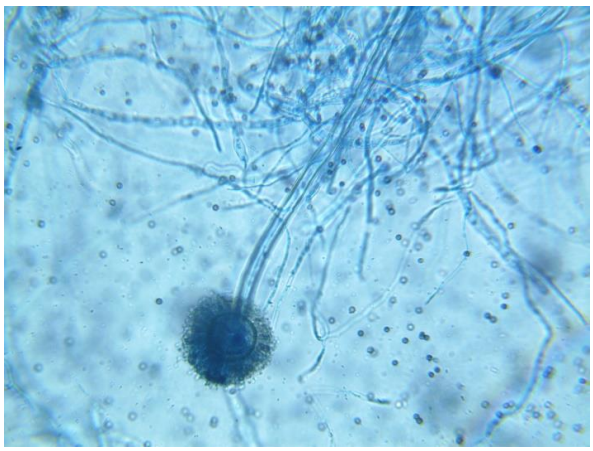
Fig. 14.0: Isolates pure culture of fungi on Slants.

4. Identification of fungal parasite

The 2 fungal species were identified as per the keys compared with Barnett and Gilbert Manual by the help of microscopic observations Fig – 15 and Table 2.0. Fungus identified were *Aspergillus flavus* and *Aspergillus niger*.



1a 1b



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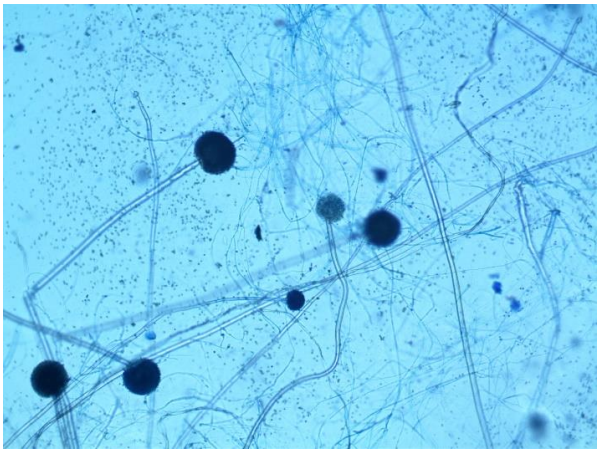

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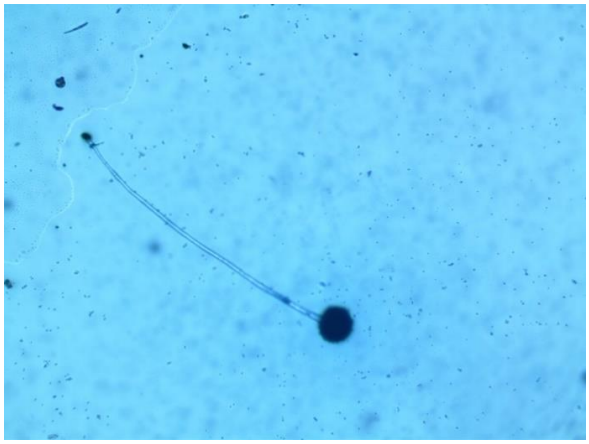

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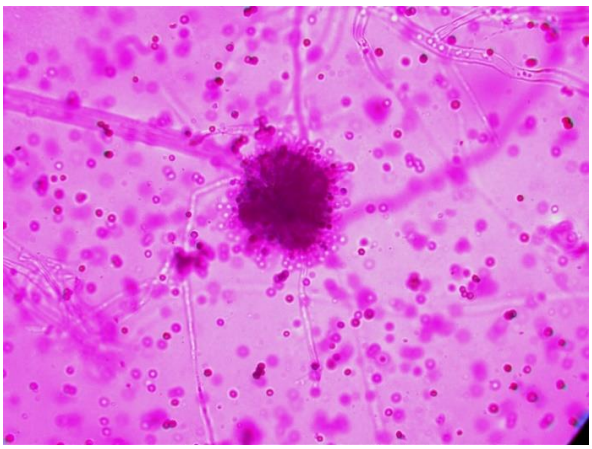

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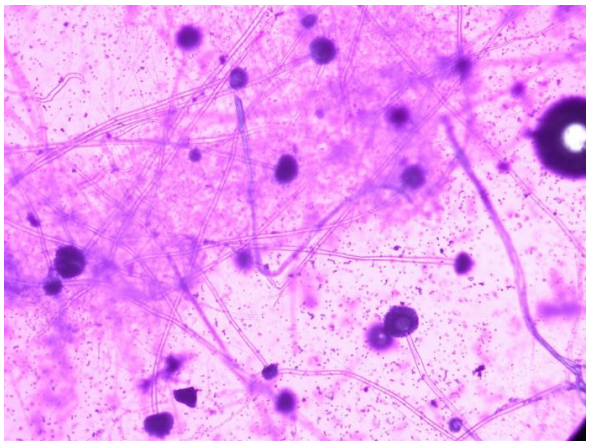

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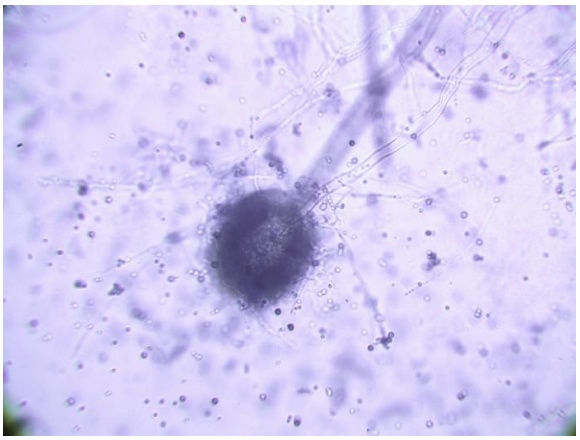

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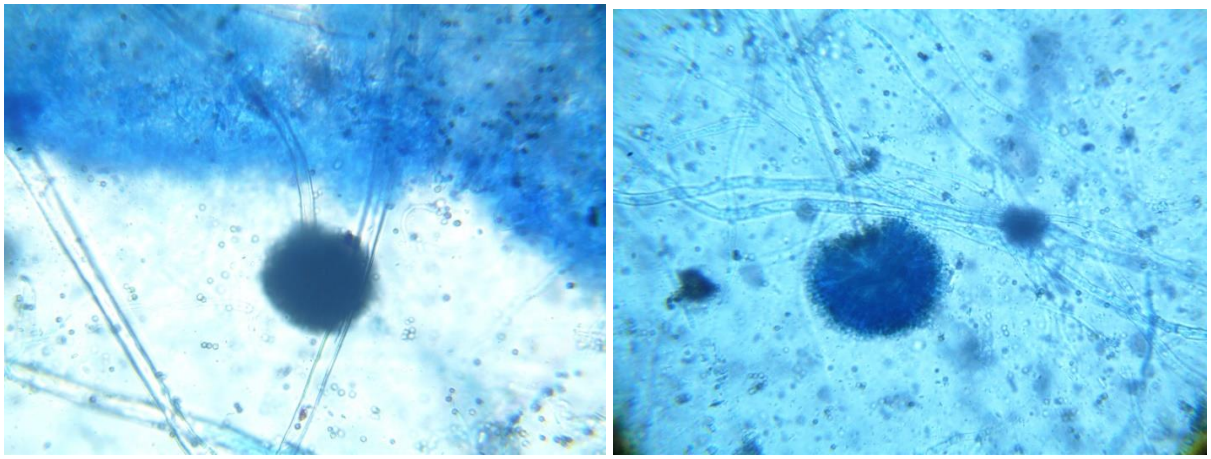


5a 5b

Fig. 15.0: Observation of different isolates of fungal slides morphological characters under the microscope.

1a, 2a, 3b, 4b and 5b slides are *Aspergillus niger*

1b, 2b, 3a, 4a and 5a Slides are *Aspergillus flavus*

Table 2.0: Morphological Characters.

Characteristics	**Isolate 1**	**Isolate 2**
Hyphae **1.Septate** **2.Aseptate**	Septate	Aseptate
Sporangiospores **1.Formed** **2.Not formed**	Formed	Formed
Oospores **1.Formed** **2.Not formed**	Not formed	Formed
Hyphae **1.With clamp connection** **2.Without clamp connection**	With clamp connection	With clamp connection
Spores **1.Formed** **2.Not formed**	Not formed	Formed
Spores **1.Ascospores formed** **2.Basidiospores formed** **3.Conidia formed**	Ascospores	Ascospores
Colony color	Black	Green
Conidiophore color	Hyaline	Hyaline

Isolate 1 and 2 is confirmed as *Aspergillus niger* and *Aspergillus flavus* as by studying the Table 2.0

5. Infestation of host plant with pathogens (*A. niger* & *A. flavus*)

The different isolates of *A. niger* and *A. flavus* were grown in suspension media to isolate their respective spores to make inoculum for infestation of healthy *Capsicum annuum* plants. The inoculum were prepared for 1 $x10^5$ spores per ml in 2% starch solution that provide stickiness for spores over healthy *Capsicum annuum* leaves and also provide the sufficient moisture for the development of mycelium which penetrate inside the epidermal cells and causes pathogenesis to the host plants.

Table 3.0: Inoculum preparation at a conc 1 x 10^5 spores per ml for different fungal isolates.

Tube No.	No. of Spores (x)	No of Spores ($\frac{x}{5}$ x10^5)	1 x 10^5 Spores per tubes		
			Spores/ml	DW/ml	Final volume of spores per ml
T1a	2+2+2+2+2=10	2 x 10^5	1	1	1 x 10^5
T1b	2+4+3+3+2=14	2.8 x 10^5	1	1.8	1 x 10^5
T2a	3+3+4+4+3=17	3.4	1	2.4	1 x 10^5
T2b	4+4+3+3+2=16	3.2	1	2.2	1 x 10^5
T3a	4+5+4+3+4=20	4	1	3	1 x 10^5
T3b	3+5+4+5+3=20	4	1	3	1 x 10^5
T4a	4+4+6+4+5=23	4.6	1	3.6	1 x 10^5
T4b	8+5+5+6+5=29	5.6	1	4.6	1 x 10^5
T5a	3+3+5+4+3=21	3.6	1	2.6	1 x 10^5
T5b	5+4+5+3+4=21	3.6	1	2.6	1 x 10^5

6. Biochemical screening of metabolite of control and infested plant

The infected leaves were processed for the extraction of tissue fluid under chilling condition (4°C) for the test of bioactive compounds presents in tissue extract. 5 gm of infected tissue sample were first surface sterilized with Sodium hypochloride and wash thrice with DW and then the sample was macerated in PBS buffer (pH 7.0). The macerated samples are then centrifuged at 1500 rpm for 20 min under chilling condition. Supernatant was used for the estimation of bioactive compounds and for enzymes. (Table 4.0 and Fig16.0 & 17.0)

Table 4.0: Biochemical Test.

Test Tube	Saponin Test/ Foam Test	Alkaloid Test (Mayer's Reagent)
Control	0	+
T1	+++	++

T2	**-**	**+**
T3	**+**	**+**
T4	**++**	**++**
T5	**++**	**+++**
T6	**+**	**+**
T7	**+**	**++**
T8	**+**	**+**
T9	**++**	**+++**
T10	**+**	**++**

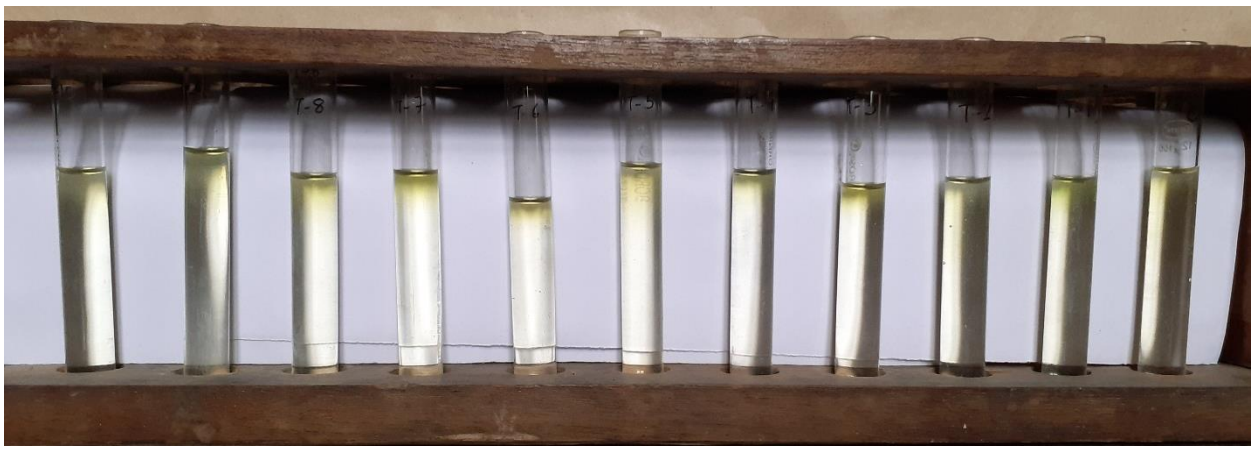


Fig. 16.0: Alkaloid Test.

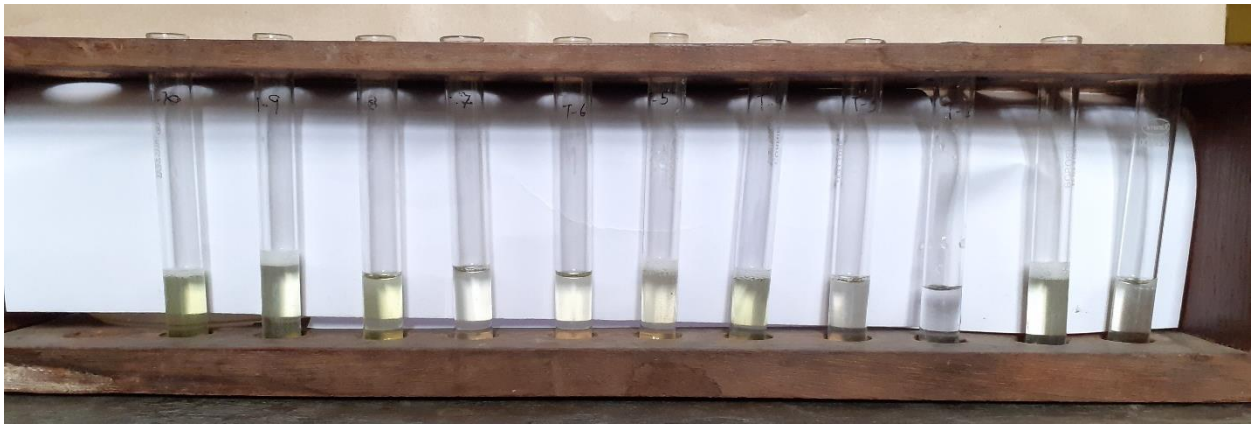


Fig. 17.0: Saponin Test.

7. Screening of host anti-parasitic or anti-fungal enzymes: Cellulase and Pectinase

All the fungal isolates showed positive result regarding the cellulase and pectinase enzyme with varied degree of activity (Table – 5.0). Both cellulase and pectinase are hydrolytic enzymes active upon the polymeric carbohydrates, cellulose and pectin present in the fungal cell of phycomycetes and other groups of fungi having chitin as main cell wall material. Therse enzymes are produced by host plant tissues as a biochemical defense against fungal pathogens.

Table 5.0: Sensitivity test of Cellulase and Pectinase.

Test Tube	Cellulase Test	Pectinase Test
Control	0	+
T1	++	++
T2	+	++
T3	+++	+
T4	+	++
T5	++	++
T6	+	++
T7	++	++
T8	+	+++
T9	++	++
T10	+++	+

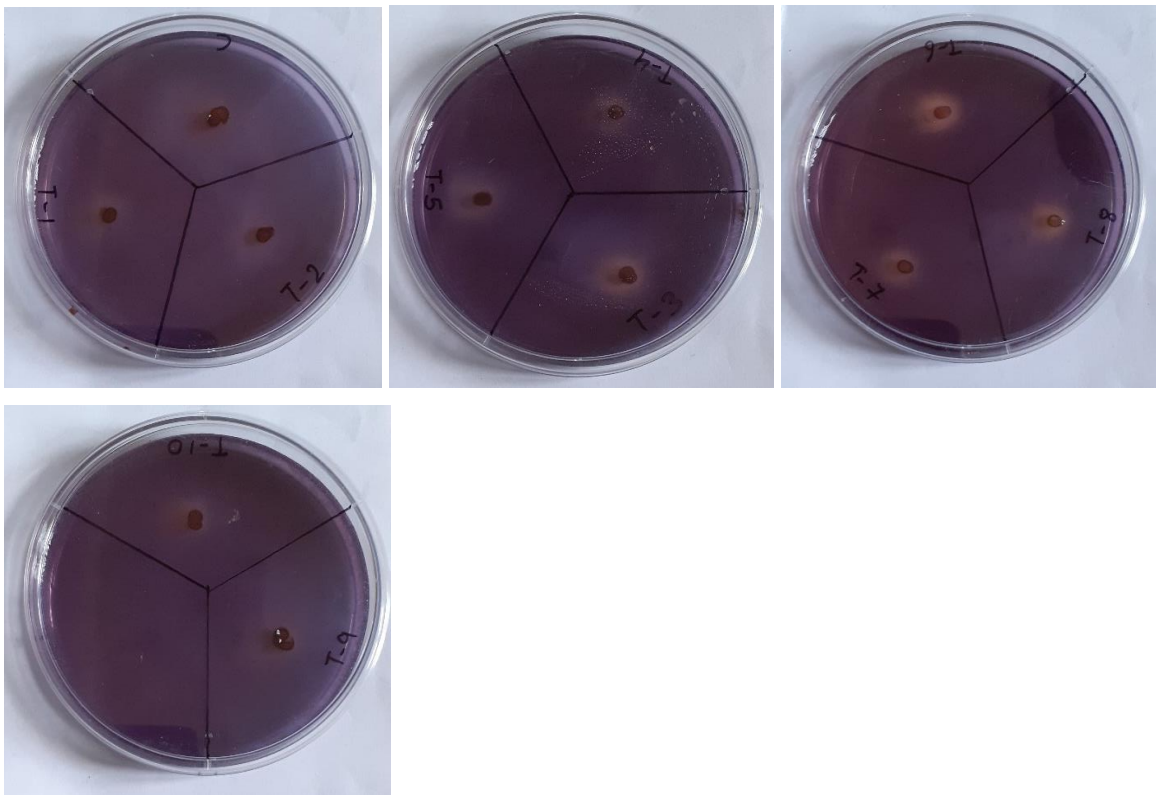
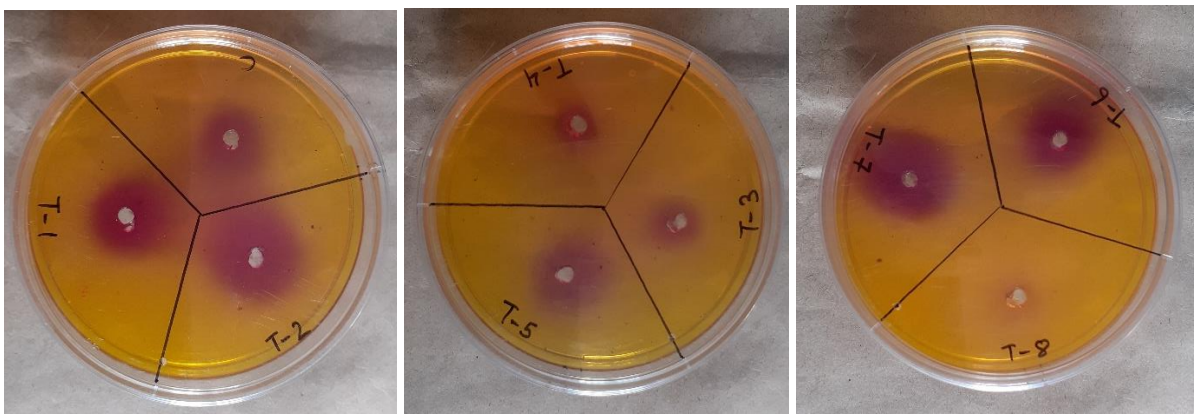


Fig. 18.0: Cellulase Test.



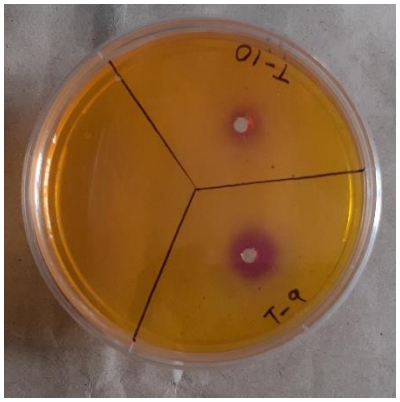


Fig. 19.0: Pectinase Test.

8. Screening of Short Peptide in host tissues

All the 10 isolates were used for the infestation of healthy *Capsicum annuum* plants to estimate the production of short peptides as a PR-proteins. These short peptides are produced by the transitional expression of post genomic DNA during pathogenesis. The pathogen triggers the signaling pathway for the production of short peptides having antipathogenic activity. The results shows that the presence of 10kDa peptides in all the infected tissues with higher expression in 2nd isolates T2 and least expression in T5. Similarly short peptide of about 5kDA is observed in 9 isolates while T 10 isolates did not show the presence of 5kDa band. Therefore both *A. niger* and *A. flavous* evokes the transitional pathway for the production of short peptides.

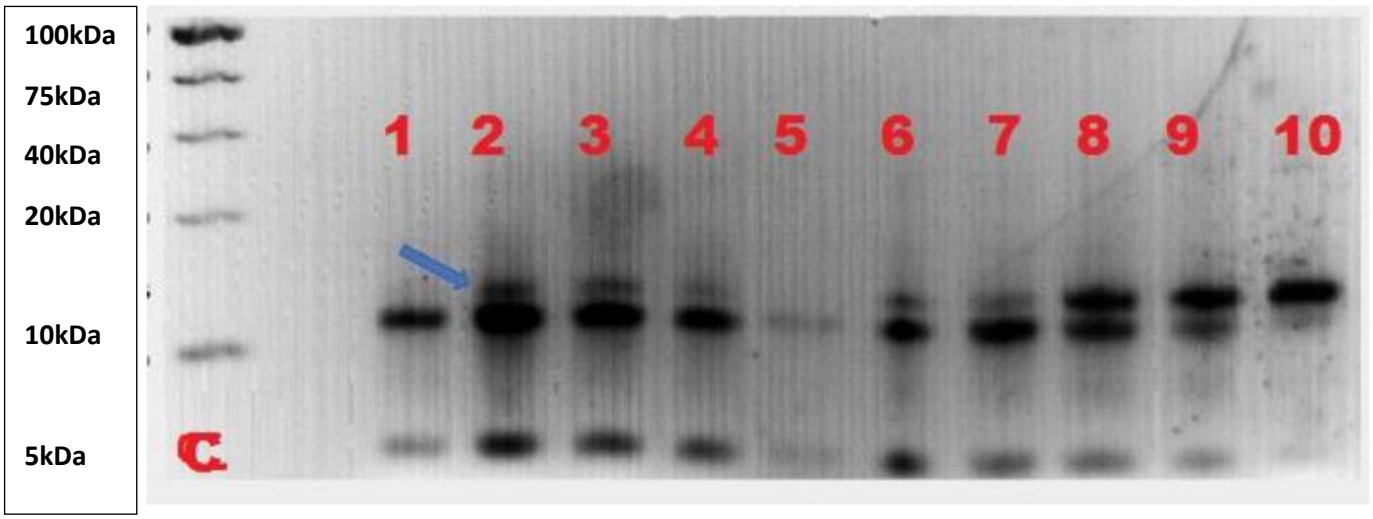


Fig. 20.0: Electrophoretic bands of short peptides.

CONCLUSION

Host parasite relationship occur as a result of prolonged evolution associations between organisms that is organisms developing or living with each other in the same environment for

a long time. The host is plant is selected as *Capsicum annuum L.* for the experimentation. The pathogens were isolated from the ex-plant which is taken from the infected leaves of *Capsicum annuum,* then the isolation on the PDA media followed by colony examination after purification by performing pure culture technique. When pure culture was obtained then the fungal species were identified that was grown on the slant by the help of morphological characteristics like hyphae, sporangiospores, spore, etc. of the fungi then resultant fungi is *Aspergillus* genus and the two species of *Aspergillus* were found that is *Aspergillus niger* and *Aspergillus flavus*. When these two species of aspergillus were identified then it infested on the plant by taking the equal amount of the pathogens by the process of spore counting method. Reading was taken at a regular interval of three days, after taking approx. ten reading the infested plant leaves were taken and grind it by mixing it with buffer of pH 7 that helps in preserve as a buffering agent of plant extract. By centrifugation method separate the supernatant and keep it safe on another Eppendorf tube, then the further biochemical test was performed like alkaloid test, saponin test, etc. In the alkaloid test we find that T2, T3, T6, T8 shows the minimum amount and T5, T9 shows the maximum amount of alkaloid in the infested plant. In the saponin test we find that T1 shows the maximum amount and the T2 shows the negative results.

Then further anti-parasitic or antifungal enzyme that is cellulose and pectinase test were perform, in the cellulose test T3, T10 shows the maximum and T2, T8 shows minimum clearance zone respectively on the agar mix with starch solution plate. This test shows the plant is under stress condition and the amount of cellulose producing under stress condition. In the pectinase test T8 shows maximum and T3, T4 shows minimum action of pectinase activity, this both the test shows the antifungal activity of plants when it is in under stress condition.

When the plant is under the stress condition it starts to produce a PR-proteins (Pathogenesis-related proteins) this is a type of protein which has ability to invade the fungal pathogens or it acts like a toxin for pathogens. In this the fifth plant shows the minimum and first plant shows the maximum number of short proteins, this conclude that plant fifth has unable to invade the pathogens.

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