4. Experimental

4.1: Introduction:

North Bengal is situated in between the mighty Himalayas and the sacred river Ganga. North Bengal stretches to the east up to the border of Bangladesh & Assam and to the west up to Bihar & Nepal. Production of tropical and subtropical fruit crops is possible due to its ecological conditions. Major horticultural crops of North Bengal are orange, tomato and pineapple etc. During the past years, there has been a great development in cultivation and production of horticultural products. As tropical and subtropical fruits are perishable due to their characteristic shapes, structure, high water content, appearance, physiological characteristics, and growing conditions, the harvested losses are also very high. Research and application of technology for storage, transport, and packaging of fruits after harvest are behind production growth in this region and many tropical and subtropical fruits have not been studied and handled adequately.

In the present study, three different fungal pathogens and three different bacterial pathogens of fruits and vegetables (orange, tomato, and pineapple) were selected to control by the antagonists and botanicals. Initially the selection have been made from 37 fungi and 76 bacteria isolated from 9 different vegetables (potato, brinjal, tomato, cucumber, papaya, carrot, bean, peas, onion) and 6 different fruits (pineapple, orange, banana, apple, mango, guava). Details of the isolation procedures have been described in materials and methods (section: 3.2). The results of the initial screening have been presented in the table (4.1). Post harvest diseased fruits and vegetables were collected from different markets of North Bengal. After selection of the six pathogens it was necessary to confirm their pathogenicity in different varieties of their hosts. Hence pathogenicity of the pathogens was performed on some of the cultivated varieties of the respective hosts. From the pathogenicity results susceptible fruit varieties against each pathogen was selected for future studies. The results of the pathogenicity of the selected three pathogens have been presented in the section 4.9.

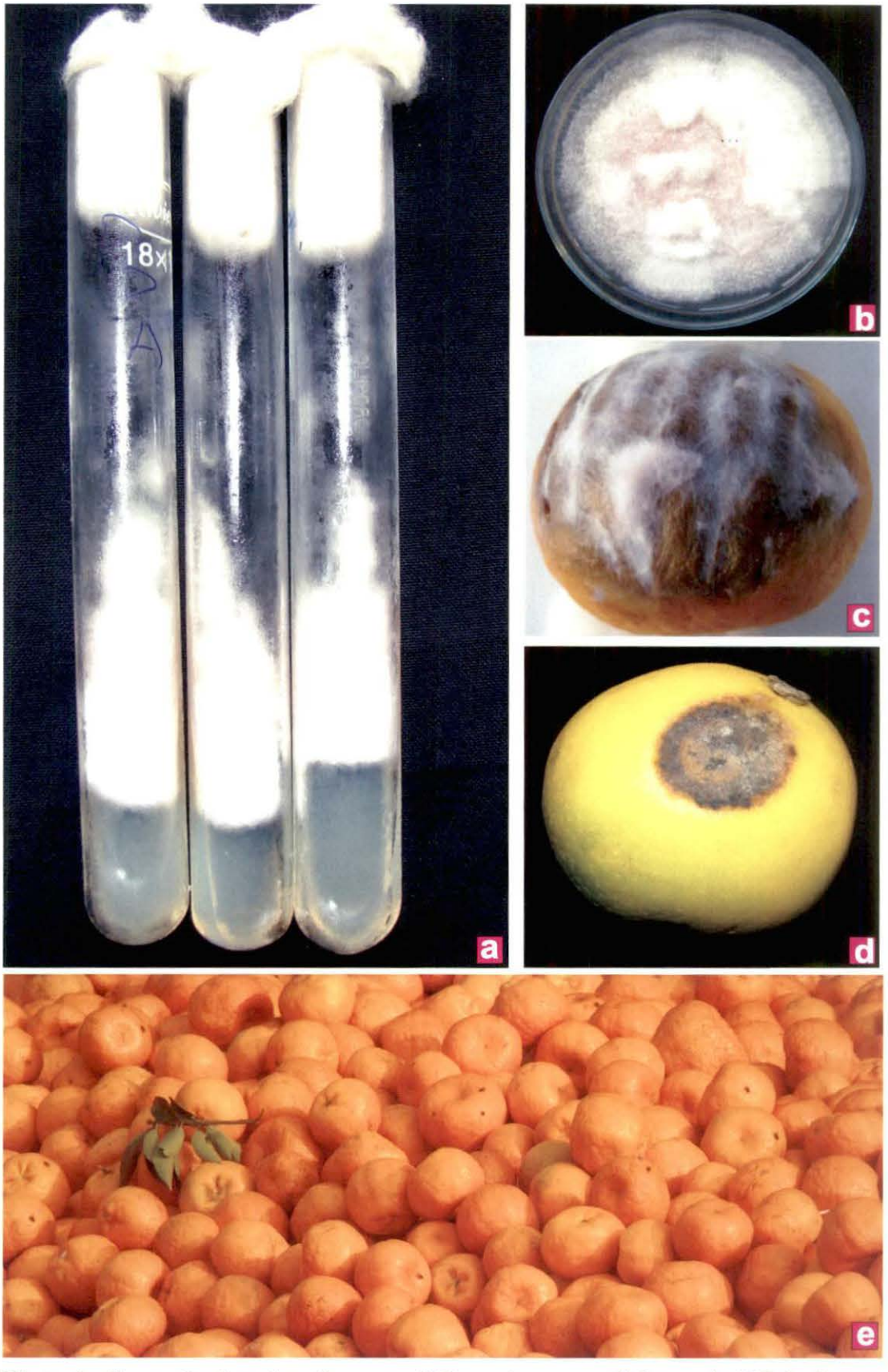


Plate 1: fig. a: Isolated pathogen of *Fusarium moniliforme* in PDA slants; **fig. b:** Sporulated culture of *F. moniliforme* in PDA plate; **fig. c:** Inoculated orange by *F.moniliforme* **fig.d:**Naturally infected orange by *F. moniliforme* **fig. e:** Harvested orange at Garubathan, Jalpaiguri.

4.2: Characterization and identification of fungal pathogens

Isolated fungal pathogens were grown in potato dextrose agar (PDA) media as described in details in material and methods section: 3.2.1.1. From diseased fruits and vegetables, a portion of the affected tissue was picked up by a long sterile forceps aseptically and inoculated in the PDA. The fungal cultures thus obtained were sub-cultured and grown for seven days for good sporulation. The morphological characters of the three fungi were studied in details in slants and petriplates. Microscopic studies were performed following lacto-phenol-cotton blue staining.

Table 4.1: Post harvest diseased fruits and vegetables of North Bengal studied.

Fruits and vegetables	No. of isolated bacteria	No. of isolated fungi
Potato	6	2
Brinjal	4	3
Tomato	7	4
Cucumber	5	2
Papaya	3	2
Carrot	2	3
Bean	3	2
Pees	2	1
Onion	5	3
Pineapple	7	3
Orange	8	5
Banana	6	3
Apple	5	2
Mango	7	3
Guava	6	2

4.2.1: Studies of morphology of *Fusarium moniliforme*

Fusarium moniliforme, isolated from orange were white in colour. In agar culture fungus grows as white mycelial mat. With maturity, a tinge of pink colour was found (Plate 1, fig b). The hypha is septate and branched when young. With advancement of growth a tinge of pink colour appears which becomesdarker at maturity.

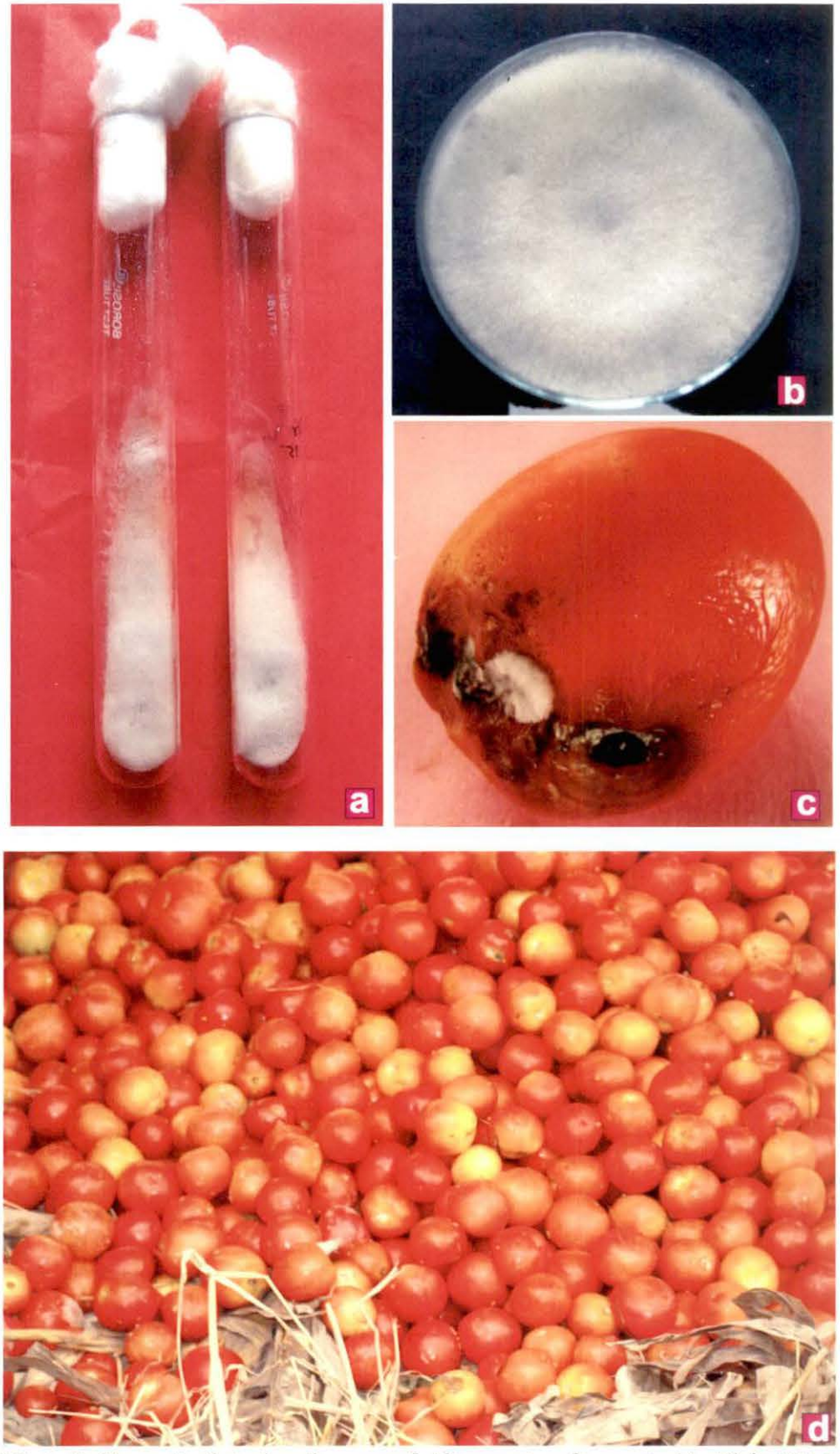


Plate 2: fig.a: Isolated pathogen of *Alternaria alternata* (AaT) in PDA slants; **fig.b:** Sporulated culture of *A.alternata* (AaT) in PDA plate; **fig.c:** Naturally infected tomato by *A.alternata* (AaT)**fig.d:** Harvested tomato at Haldibari,Coochbehar

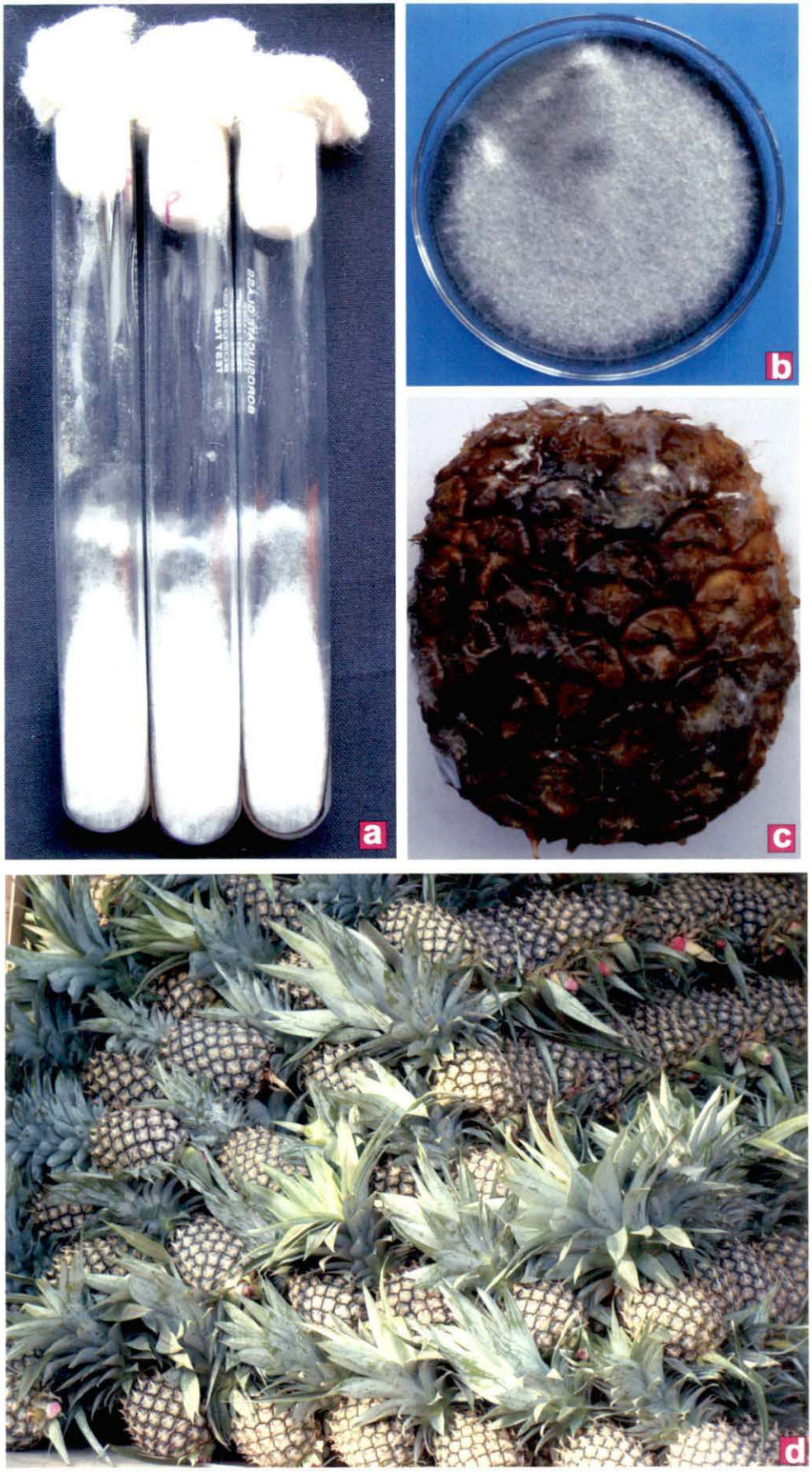


Plate 3: fig.a: Isolated pathogen of *Alternaria alternata* (AaP) in PDA slants; **fig.b:** Sporulated culture of *A.alternata* (AaP) in PDA plate; **fig.c:** Naturally infected pineapple by *A.alternata* (AaP) **fig.d:** Harvested pineapple at Bidhannagar, Darjeeling.

For microscopic observations, mycelia were taken in microscopic slide from pure culture and stained with cotton-blue in lacto-phenol. The slides were mounted with cover glass, sealed and observed under microscope. Two different types (micro and macro conidia) of conidia were found. The micro conidia were born terminally on short hyphae. They were small elliptical or curved 2 celled structures. Macro conidia were formed on mature hyphae. Macro conidia were long, curved pointed at the top and with 3 to 4 septa.

4.2.2: Studies on morphology of *Alternaria alternata* (isolate AaT)

Alternaria alternata (AaT) isolated from tomato did not turn completely black but blackish mycelia were intermingled with white mycelial mat. Mycelia when grown on tomato is white but at later stages of infection it turns black and visible on damaged portion of the host tissue (Plate 2,fig c). *Alternaria alternata* (AaT), the isolated pathogen of tomato, was grown in PDA and PDB media and morphology of the pathogen along with mycelial growth was observed. Profuse sporulation of the fungi was observed in PDA.

For microscopic observations, mycelia were taken in microscopic slide from pure culture and stained with cotton-blue in lacto-phenol. The slides were mounted as stated earlier. Immature mycelia were hyaline in colour but on maturity it became gray in colour. Conidia of the fungus were obclavate to beaked and brownish in colour having transverse and longitudinal septa. Conidia were produced from simple septate conidiophores in simple or branched acropetal chains. The length and breadth of mature conidia were 10-20 μm and 6-8 μm respectively. The diameter of the mature hyphae ranged between 3-5 μm.

4.2.3: Studies on morphology of *Alternaria alternata* (isolate AaP)

Hyphae when growing on pineapple is white but damaged skin of pineapple turns black from characteristic golden yellow colour of the pineapple fruits of north Bengal. *Alternaria alternata* (AaP), the isolated pathogen of pineapple, was grown in PDA and PDB media and morphology of the pathogen along with mycelial growth was observed. Profuse sporulation of the fungi was observed in PDA (Plate: 3, fig. b). *Alternaria alternata* (AaP) grows as white mycelia but with maturity it becomes blackish when grown on pineapple fruits.

For microscopic observations, mycelia were taken in microscopic slides from pure culture and stained with cotton-blue in lacto-phenol. The slides were mounted with cover glass and were observed under microscope. Fungal mycelia were profusely branched and septate and light brown in colour. Spores or conidia were produced exogenously on conidiophores. Conidiophores were not recognisable. Conidia were large and dark coloured, several celled and beaked. The septa dividing the spores into cells were both transverse and vertical. The length and breadth of mature conidia were 8-16 μm and 4-6 μm respectively. The diameter of the mature hyphae ranged between 3-5 μm.

4.3: Morphological and biochemical tests for characterisation of the isolated bacterial pathogens (*Xanthomonas* sp., *Pseudomonas syringae*, and *Erwinia* sp.) and antagonist bacteria (*Lysinibacillus sphaericus* and *Pseudomonas* sp.)

Bacterial pathogens and antagonists were grown in nutrient agar (NA) media. The bacterial cultures thus obtained were sub-cultured and grown in NA and NB medium. The morphological characters of the three bacteria were studied in details following appropriate bacterial staining or tests as required for specific results. Details of the procedure have been described in materials and methods (section: 3.3.1.). Similarly biochemical tests were also performed with specific tests as mentioned in materials and methods in section: 3.3.2.

4.3.1: Morphological characterisation of isolated bacterial pathogen and antagonist

For morphological characterisation of bacteria (that were originally isolated from the diseased portion of fruits and vegetables like orange, tomato, pineapple and from soil), a number of specific studies in plates and slants were performed. In some cases slides were prepared and observed microscopically following specific staining. Details of the techniques have been mentioned in the materials and methods sections: 3.3.1. The results have been presented in table (4.2) and (4.5). All the isolates of bacterial pathogens were Gram negative and rods in shape.

4.3.2: Biochemical characterisation of isolated bacterial pathogens and antagonists

For biochemical characterisation of the bacteria various tests were performed. Details of the techniques have been mentioned in the materials and methods sections: 3.3.2. The results have been presented in table (4.2) to (4.5).

All the selected pathogens tested were aerobic as they grew in the upper layer and middle layer of the medium. No submerged growth was visible.

Table4.2: Morphological and cultural characteristics of three bacterial pathogens (*Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp.).

Character of study		*Xanthomonas* sp.	*Pseudomonas syringae*	*Erwinia* sp.
Shape		Rod	Rod	Rod
Size		0.3-0.7X1.2 µm	0.5- 0.8× 1.3µm	0.8-2.0X0.4-0.6µm
Gram nature		Gram negative Aerobic in nature	Gram negative Aerobic in nature	Gram negative Aerobic in nature
Motility		Motile	Motile	Motile
Agar plate character	Growth	Moderate	Fast growing	Moderate
	Form	Flat	Irregular	Flat
	Surface	Smooth	Smooth	Smooth and mucoid
	Elevation	flat	Flat	Raised
	In broth	Turbid	Turbid	Turbid with white sediment
	Colour	Whitish gray/off white	Whitish	Whitish grey

Pseudomonas syringae was cultured in *'Pseudomonas* Agar media' and fluorescence was also observed. Bacterial strains were streaked on *Pseudomonas* Agar (for Fluorescein) plates and incubated at 30°C for 24 hours. The plates were then observed under UV light for presence of fluorescence.

Table4.3: Biochemical characteristics of three selected bacterial pathogens (*Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp).

Biochemical Characteristics	Bacterial strains		
	Xanthomonas sp.	*Pseudomonas syringae*	*Erwinia* sp.
Pigment production	-	-	-
Indole production	-	-	-
Methyl Red	-	-	+
VP test	-	-	+
Citrate utilization	+	-	+
Acid production in TSI agar	+	+	+
Gelatin hydrolysis	+	+	+
Oxidase	+	-	+
Catalase	+	+	+
Urease	-	-	-
Nitrate reduction	-	+	-
Starch hydrolysis	+	+	-
Casein hydrolysis	-	+	+
Phenylalanine decarboxylase	-	-	-
Tween 80 hydrolysis	+	+	+
DNase production	-	+	-
Acid from Glucose	+	+	+
Acid from Fructose	+	+	+
Acid from Sucrose	+	+	+

Contd.....

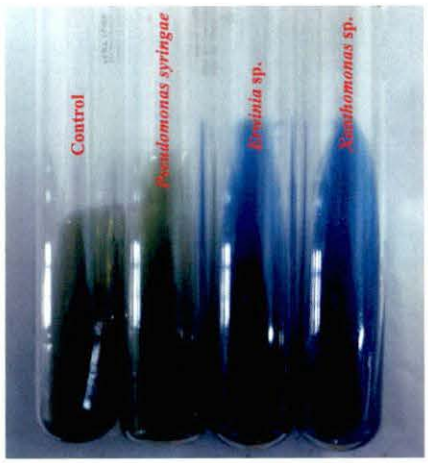
Table4.3 (Contd.): Biochemical characteristics of three selected bacterial pathogens (*Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp.).

Biochemical Characteristics	Bacterial strains		
	Xanthomonas sp.	*Pseudomonas syringae*	*Erwinia* sp.
Acid from Dulcitol	-	-	+
Acid from Galactose	+	+	+
Acid from Rhamnose	-	-	-
Acid from Raffinose	-	-	+
Acid from Maltose	-	-	-
Acid from Lactose	+	-	+
Acid from Xylose	-	-	+

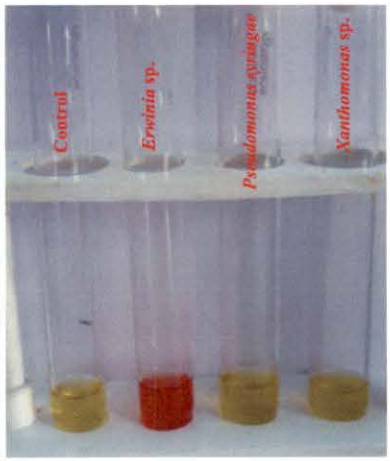
'+': positive '-': negative

On the basis of the characters (morphological and biochemical) presented in different sections above, three bacterial pathogens were identified in the laboratory following Bergey's Manual of Determinative Bacteriology (Holt *et al*, 1994). Thus, the bacteria were identified as *Xanthomonas* sp. (isolated from orange), *Pseudomonas syringae* (isolated from tomato) and *Erwinia* sp. (isolated from pineapple).

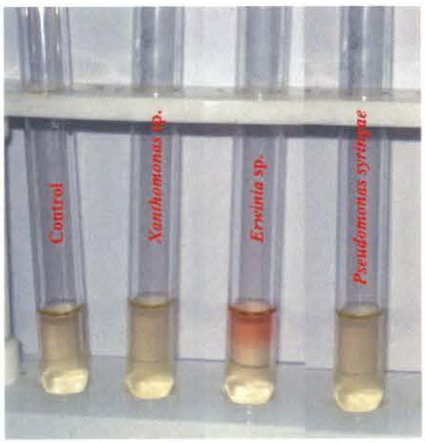
The characters common to all the three bacteria were production of acid and gas from glucose, fructose, sucrose, galactose (Plate: 4). Tween 80 hydrolysis test were performed for lipolytic activity of the bacteria. All the three bacteria were lipolytic activity positive and they produced acid in TSI agar. All the three bacteria were catalase positive but they showed negative results in pigment production, indole production and phenylalanine decarboxylase tests. All the three bacteria were also negative in production of acid from rhamnose, xylose and urease.



Citrate utilization test



Methyl Red test



Voges Proskauer test

Acid Production from Glucose

Acid Production from Sucrose

Triple Sugar Iron test

Plate 4: Results of some biochemical tests performed for pathogenic bacteria.

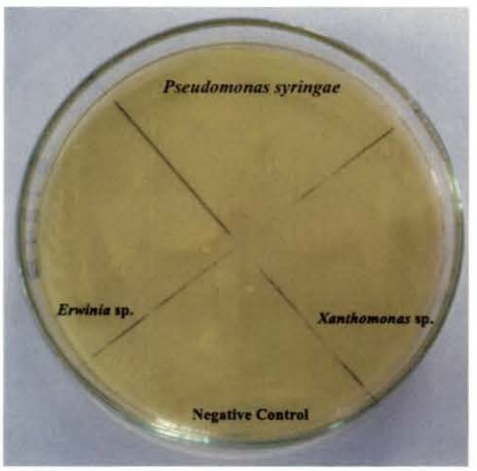
Out of the three bacteria tested *Xanthomonas* sp. *and Erwinia* sp. was insensitive to nitrate reduction and deoxyribonuclease production. But showed positive results in citrate utilization (Plate: 5).

Only *Erwinia* sp. showed the positive results in acid production from xylose, Raffinose, Dulcitol, Methyl Red and VP test. *Erwinia* sp. also showed negative results in case of Starch hydrolysis (Plate: 5).

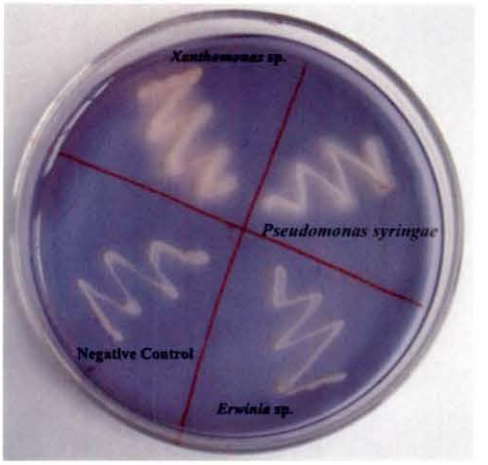
Pseudomonas syringae was found negative in methyl red test, VP production, citrate utilization, urease and acid from lactose in contrast to other two bacteria. *Pseudomonas sp* also produced acid from maltose in contrast to other two bacteria tested. (Bergey's manual).

Table4.4: Morphological and cultural characteristics of two bacterial antagonist (*Lysinibacillus sphaericus, Pseudomonas* sp.)

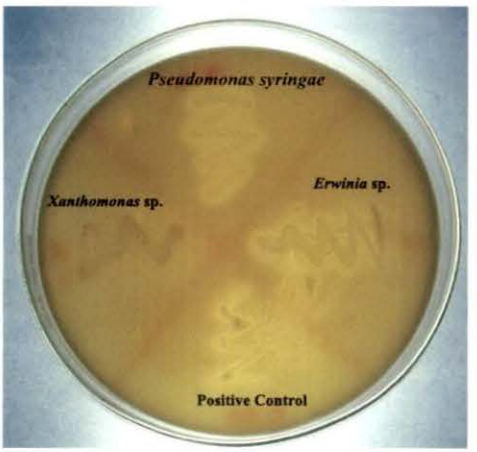
Character of study		*Lysinibacillus sphaericus*	*Pseudomonas* sp.
Shape		Rod	Rod
Gram nature		Gram positive Aerobic in nature	Gram negative Aerobic in nature
Motility		Motile	Motile
Agar plate character	Growth	Moderate	Fast growing
	Form	Flat	Irregular
	Surface	Smooth	Smooth
	Elevation	Flat	flat
	In broth	Turbid	Turbid
	Colour	off white	Whitish



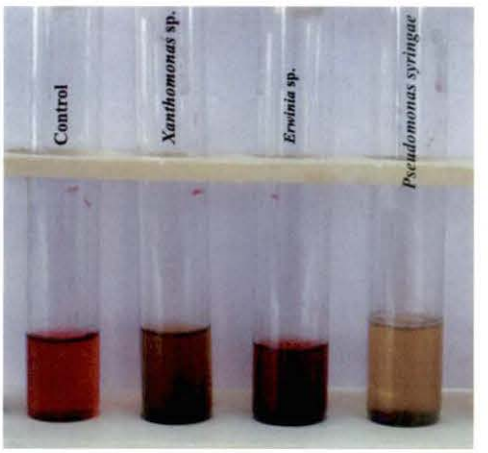
Urease Production test



Starch Hydrolysis test



Casein Hydrolysis test



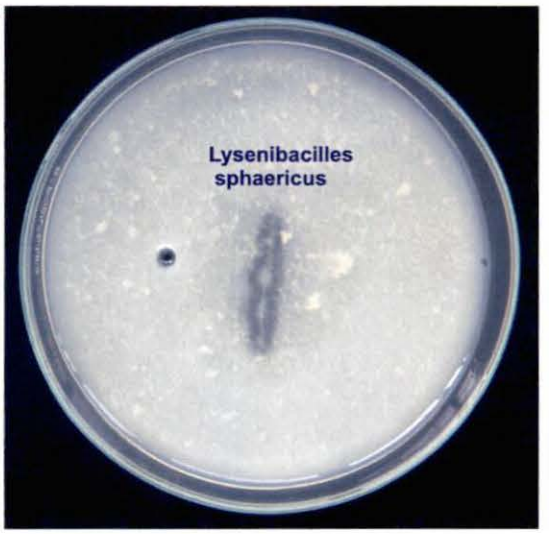
Nitrate Reduction test

Plate 5: Results of some biochemical tests performed for pathogenic bacteria.

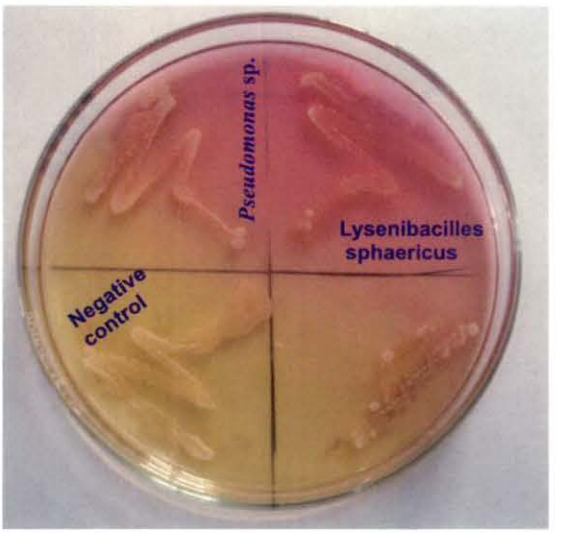
Table4.5: Biochemical characteristics of two selected bacterial antagonist (*Lysinibacillus sphaericus* and *Pseudomonas sp.*)

TESTS	*Lysinibacillus sphaericus* (SB1)	*Pseudomonas* sp. (SB2)
Indole	-	-
Methyl red	-	-
VP	+	+
citrate	+	+
gelatinase	+	-
Caseinase	+	+
urease	+	+
Catalase	+	+
oxidase	+	+
phosphatase	-	-
Chitinase	-	-
cellulase	+	-
Acid production in TSIA	-	+
Starch hydrolysis	+	-
Pectinase	-	-
ONPG	+	+
Siderophores	+	+
Nitrate reduction	+	+
Phenyl alanine decarboxylase	-	-
Sugar fermentation-		
Glucose	+	+
Maltose	+	-
Lactose	-	-
Galactose	-	-
Fructose	+	+
Sucrose	+	+
Raffinose	-	+/gas
D-Arabinose	+	-
xylose	+	+

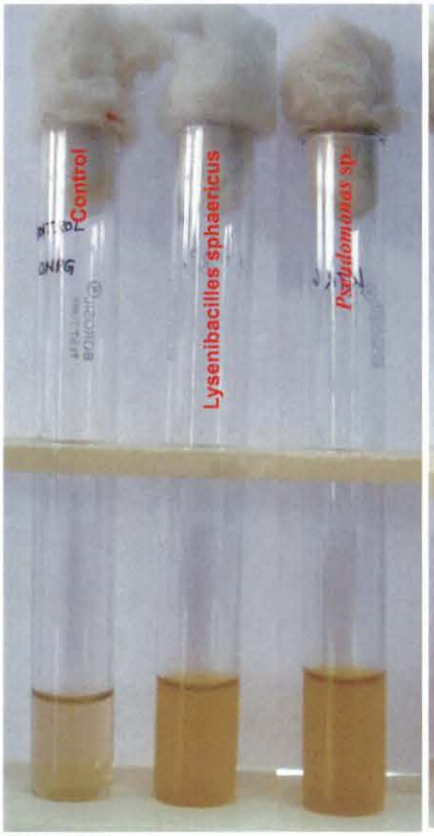
Similarly morphological biochemical characteristics of antagonistic bacteria (Table: 4.4 and 4.5) were also analysed and which led us to identify the organisms as SB1 (seems to be *Bacillus* sp.) and SB2 (*Pseudomonas* sp.) SB1 was further analysed for 16S rDNA studies. The results of 16S rDNA studies have been presented in section: 3.6.5; plate: 6.



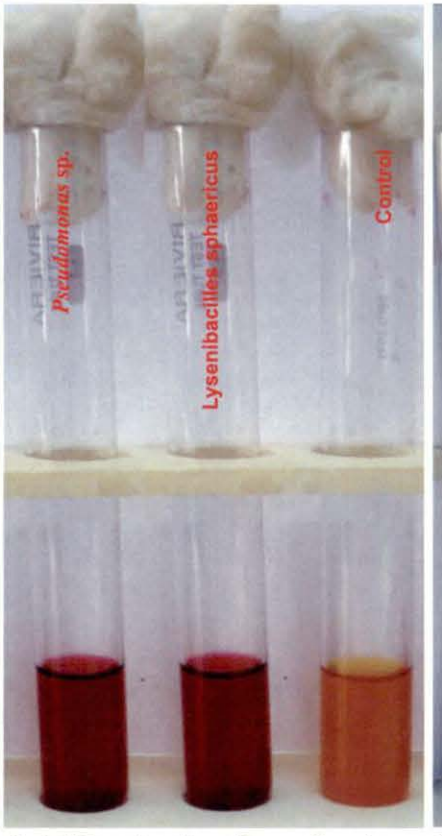
Caseinase production test



Urease production test



ONPG test



Acid Production from fructose

Citrate utilization test

Plate 6: Results of some biochemical tests performed for antagonistic bacteria.

4.4: Screening of potential antifungal botanicals against *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)

To develop a complete eco-friendly method for controlling fungal and bacterial pathogens of diseased fruits and vegetables botanical extracts were tested for their antifungal efficacy *in vitro* following spore germination bioassay. Several workers have successfully utilized extracts of numerous plants for controlling post harvest diseases. Several plants were collected from different parts of sub-Himalayan West Bengal and study was undertaken to screen the potential antifungal activity against the pathogen. The procedures of aqueous and 50% ethanolic extracts preparation of the collected plants have been discussed in materials and methods (sections: 3.5.1 & 3.5.2). Different techniques of bioassays viz. spore germination bioassay, poisoned food bioassay, agar cup bioassay and TLC-plate bioassay have also been discussed in materials and methods (sections: 3.5.5, 3.5.6, 3.5.7 & 3.5.9 respectively).

4.4.1: Spore germination bioassay to screen potential botanicals for controlling *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)

To screen potential antifungal activity, fifty plants were collected from different parts of sub-Himalayan West Bengal. Extracts of the leaves (both aqueous and 50% ethanolic) were prepared and used for their antifungal properties against *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP). Spore germination technique described by Suleman *et al.,* (2002) was followed for the screening. A detail of the procedures of spore germination bioassay and preparation of extracts (both the aqueous and 50% ethanolic) have been discussed in materials and methods (sections: 3.5.1 & 3.5.2). Detailed results of efficacy of plant extracts have been presented in the table (4.6). Data presented in the table were calculated on the basis of the percent inhibition of spore germination of the three different fungi in different plant extracts tested.

Table 4.6: Percent inhibition of spore germination of *Fusarium moniliforme*, *Alternaria alternata* (isolate AaT) and *Alternaria alternata* (isolate AaP) in relation to control (when control rose to 100)

Name of the plant	Family	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
		Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract
Eucalyptus citriodora	Myrtaceae	15	20	10	15	20	27
Melastoma malabathricum	Melastomaceae	82	90	90	95	90	95
Mimosa pudica	Fabaceae	90	95	80	85	82	90
Nerium indicum	Apocynaceae	40	60	45	60	75	55
Citrus aurautium	Rutaceae	8	7	3	3	0	0
Chenopodium album	Chenopodiacea e	25	35	20	30	28	22
Solanum khasianum	Solonaceae	55	60	20	23	32	12
Oxalis corniculata	Oxalidaceae	20	6	45	34	27	9
Citrus medica	Rutaceae	0	0	0	0	0	0
Datura stramonium	Solanaceae	100	100	100	100	100	100
Datura metel	Solanaceae	80	85	75	70	80	80
Elephantopus scaber	Euphorbiaceae	00	00	01	05	10	07
Tectona grandis	Verbenaceae	10	20	00	05	10	00
Parthenium hysterophorus	Compositeae	80	75	05	05	10	00
Polyalthia longifolia	Anonaceae	02	05	05	00	00	05
Borreria alata	Rubiaceae	85	90	80	90	80	85
Bambusa tulda	Poaceae	00	00	00	00	00	00
Cassia tora	Caesalpiniaceae	35	40	15	20	05	07
Catharanthus roseus	Apocyanaceae	18	42	23	30	32	16
Curcuma longa	Zingiberaceae	56	34	67	56	34	62
Caesalpenia cuculata	Caesalpiniaceae	53	49	33	40	24	25

contd...

Table 4.6: (contd.) Percent inhibition of spore germination of *Fusarium moniliforme, Alternaria alternata* (isolate AaT) and *Alternaria alternata* (isolate AaP) in relation to control (when control rose to 100)

Name of the plant	Family	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
		Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract
Cyanodon dactylon	Poaceae	00	00	00	00	00	00
Croton bonplandianum	Euphorbiaceae	05	00	00	00	00	00
Clerodendron viscosum	Verbenaceae	35	27	43	56	21	65
Xanthium strumarium	Compositae	100	100	100	100	100	100
Acalypha indica	Euphorbiaceae	09	02	11	21	09	00
Azadirachata indica	Meliaceae	39	55	25	47	53	21
Emblica officinalis	Euphorbiaceae	38	47	10	00	00	09
Eupatorium odoratum	Compositae	10	5	00	00	00	00
Anisomeles indica	Lamiaceae	00	00	00	00	00	00
Elephantopus scaber	Euphorbiaceae	85	91	79	82	56	78
Ficus religiosa	Moraceae	15	25	09	00	00	00
Ecylapha alba	Compositeae	5	10	03	05	01	00
Floganthus thyrisuiflorus	Acathaceae	10	5	00	00	00	00
Holarrhena antidysenterica	Apocynaceae	11	9	02	09	32	12
Hyptis suaveolens	Lamiaceae	21	15	43	13	23	09
Jatropha curcas	Euphorbiaceae	48	27	65	45	67	80
Lantana camara	Verbenaceae	19	44	34	21	34	61
Lagerstromia flos-reginae	Lythraceae	05	10	31	11	43	12
Amaranthus spinosa	Amaranthaceae	15	20	12	09	32	12
Manihot esculenta	Euphorbiceae	00	00	00	00	00	00

Contd...

Table 4.6: (contd.) Percent inhibition of spore germination of *Fusarium moniliforme, Alternaria alternata* (isolate AaT) and *Alternaria alternata* (isolate AaP) in relation to control (when control rose to 100)

Name of the plant	Family	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
		Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract	Aquous Extract	Ethanolic extract
Dryopteris filix-mass	Dryopteridaceae	50	60	45	55	25	29
Laenia coramendalia	Lythraceae	10	5	12	17	6	9
Chenopodium ambrosoides	Chenopodiaceae	52	63	43	49	23	29
Citrus medica	Rutaceae	00	00	00	00	00	00
Camellia japonica	Theaceae	10	00	00	00	00	00
Mykenic micrantha	Compositae	05	05	00	00	00	00
Coffea bengalensis	Rubiaceae	00	00	00	00	00	00
Crotalaria mucronata	Caesalpiniaceae	00	00	00	00	00	00

It was evident from the results (Table:4.6) that both the aqueous and 50% ethanolic extracts of *Xanthium strumarium* and *Datura stramonium* completely inhibited spore germination of *Fusarium moniliforme*, *Alternaria alternata* (AaT) and *Alternaria alternata* (AaP). Botanicals extracted from three more plants (*Melastoma malabathricum, Borreria alata and Mimosa pudica*) were also significant in controlling spore germination of the three fungal pathogens tested.

4.4.2: Antifungal effect of selected plant extracts on growth of pathogens following poisoned food technique

Poisoned food technique was followed with leaf extracts (aqueous & 50% ethanolic) of *Xanthium strumarium* and *Datura stramonium*. Details of the procedure have been described in the materials and methods (section: 3.5.6) to test their antifungal activity. Radial measurement of growth of two plant extracts was noted in table (4.7).

From the results it was clear that aqueous and 50% etahnolic leaf extracts of *Xanthium strumarium* showed significant inhibitory effect against *F. moniliforme* with 82.22% and

85.55% (plate 7: fig. a) inhibition respectively. Leaf extracts of *Datura stramonium* showed less inhibition than *Xanthium strumarium* leaf extract (plate 7: fig. b, d and f).

Table 4.7: Effect of antifungal activity of selected plant extracts on the growth of *F.moniliforme*, *A. alternata* (isolate AaT) and *A. alternata* (isolate AaP) (following poisoned food technique)

Plants	Type of extract	*Fusarium moniliforme*		*Alternaria alternata* (AaT)		*Alternaria alternata* (AaP)	
		*Radial growth (mm)	**Percent inhibition	Radial growth (mm)	Percent inhibition	Radial growth (mm)	Percent inhibition
Xanthium strumarium	Aqueous extract	16±1.47	82.22	33±1.05	63.33	19±0.55	78.88
	Ethanolic extract	13±0.57	85.55	29±0.86	67.77	17±1.37	81.10
Datura stramonium	Aqueous extract	19±0.95	78.88	39±0.91	56.66	21±1.51	76.66
	Ethanolic extract	18±1.57	80.00	34±0.97	62.22	13±1.51	78.88
CD (5%)		2.29		1.24		0.47	

Control diameter = 90 mm; Data are mean of three replications; PDA : extract = 9:1; *Data were taken after 4 days of incubation. ** Percent inhibition were calculated in relation to control (where no inhibition of growth was recorded)

4.4.3: Agar cup method for detection of anti-fungal activity by *Xanthium strumarium* & *Datura stramonium*

Xanthium strumarium and *Datura stramonium* leaf extracts were tested following agar cup bioassay technique (described in section: 3.5.7) to test their antifungal activity as well as to determine the minimum inhibitory concentrations. Radial measurement of inhibition zones of different concentrations was tabulated. It was also considered worthwhile to determine the MIC values of aqueous and bioactive fraction of the plant leaf extract of the selected test plants against the three pathogens. Different concentrations of the extracts were prepared and were poured in agar cups or wells (5mm in diameter) and the lowest concentration which showed the inhibition were considered as MIC of the respective extracts. Table (4.8) to (4.11) indicated the minimum inhibitory concentration of each plant leaf extract against *Fusarium moniliforme*, *Alternaria alternata* (AaT) and *Alternaria alternata* (AaP) [Details of the experimental procedures have been described in materials and methods section: 3.5.7].

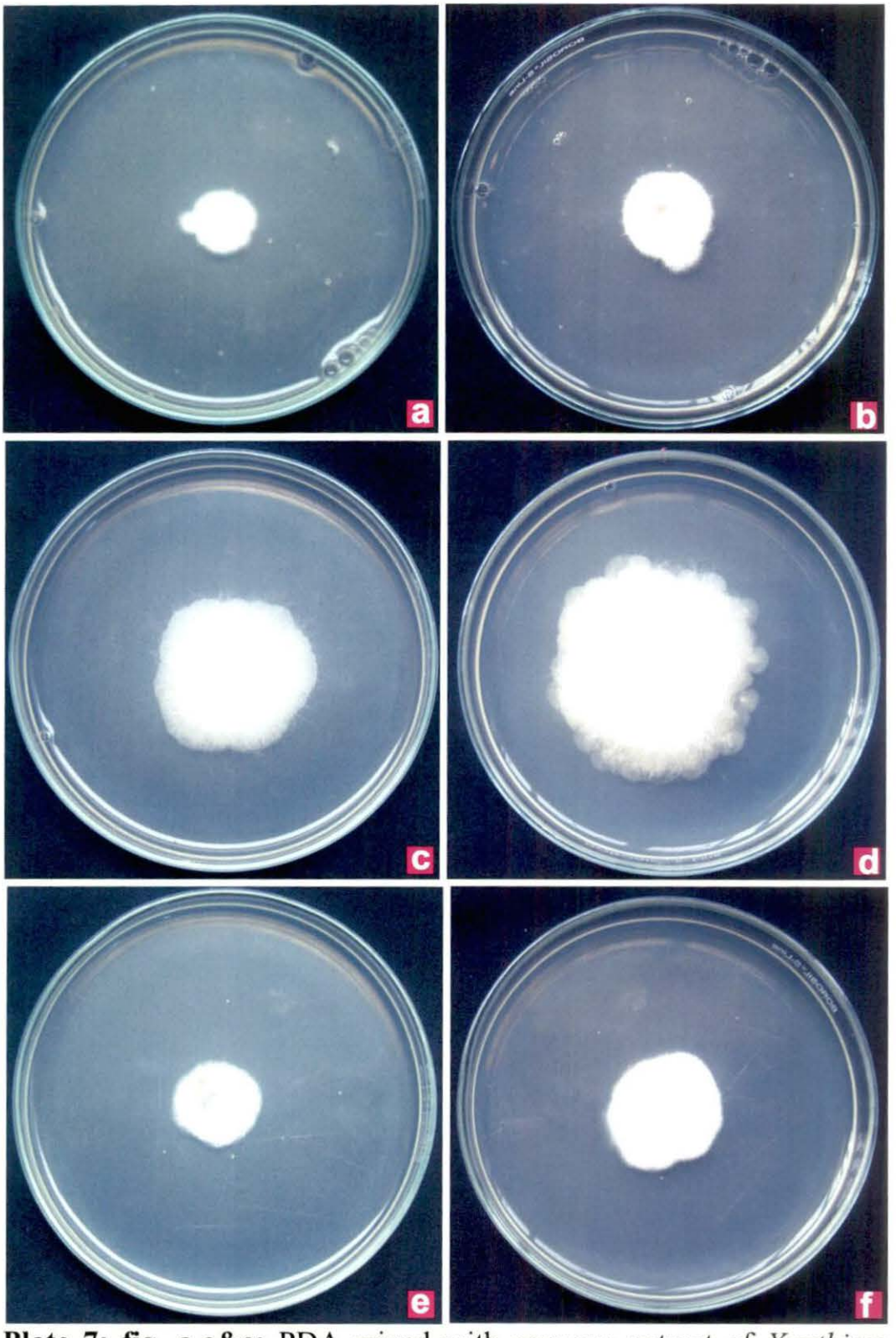


Plate 7: fig. a,c&e: PDA mixed with aqueous extract of *Xanthium strumarium* and growth of *F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP)

fig. b,d&f: PDA mixed with aqueous extract of *Datura stramonium* and growth of *F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP).

Xanthium strumarium leaf extract (aqueous) were tested against three different fungal pathogens (Table: 4.7). MIC values of *Xanthium strumarium* plant leaf extract against *F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP) were also determined. From the results it was evident that MIC values of *Xanthium strumarium* aqueous leaf extracts against *F. moliniforme*, *A. alternata* (AaT) and *A. alternata* (AaP) were 1.00 mg/ml; 1.00 mg/ml; 4.00 mg/ml, respectively.

Table 4.8: Inhibition of growth of three pathogens by different concentrations of leaf extracts of *Xanthium strumarium* (following agar cup method).

Plant extract	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)
aqueous leaf extract of: *Xanthium strumarium*	6.00	13±0.93	6.00	12±1.59	8.00	12±0.57
	4.00	11±1.31	4.00	10±1.01	7.00	11±1.10
	2.00	09±0.62	2.00	08±0.70	6.00	09±0.64
	1.00	08±0.59	1.00	07±0.29	4.00	07±0.12
	0.50	00	0.50	00	2.00	00
	control	00	Control	00	Control	00
CD5%		0.18		0.39		0.42

Data are mean of three replications

Similarly, bioactive fraction of *Xanthium strumarium* leaf extract were tested against the three different fungal pathogens (Table:4.9). The MIC values of *Xanthium strumarium* plant leaf extract (bioactive fraction) against *F.moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP) were 100 µg/ml ; 200 µg/ml ; 500µg/ml, respectively (plate 8: fig. a; plate 9: fig. a and b; plate 10: fig. a and b).

Table4.9: Inhibition of growth of three pathogens by different concentrations of bioactive fraction of leaf extract of *Xanthium strumarium* (following agar cup method).

Plant extract	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
	Extract Concentration (µg/ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)
bioactive fraction of leaf extract of: *Xanthium strumarium*	250	17±0.90	500	20±1.53	1250	13±0.40
	200	16±1.01	400	18±0.93	1000	11±0.76
	150	14±2.00	300	15±0.46	750	9±0.55
	100	13±0.90	200	12±0.40	500	7±0.40
	50	00	100	00	250	00
	control	0.00	Control	00	Control	00
CD5%		0.23		0.39		0.34

Data are mean of three replications

Datura stramonium (aqueous leaf extract) were also tested for their antifungal activity against the three selected fungal pathogens [*F. moniliforme*, *A. alternata* (isolate AaT) and *A. alternata* (isolate AaP)]. Details of the results have been presented in table (4.10). Minimum inhibitory concentrations (MIC) of *Datura stramonium* aqueous leaf extract against *F. moniliforme*, *A. alternata* (isolate AaT) and *A. alternata* (isolate AaP) were 0.50mg/ml; 2.00mg/ml ; 5.00mg/ml respectively.

Similar experiments were also performed for evaluation of antifungal activity of *Datura stramonium* leaf extract (bioactive fraction) against *F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP). Details of the results have been presented in table 4.11. From the results it was evident that all the three fungi tested showed reduced growth in presence of bioactive fraction of *Datura stramonium* leaf extracts (almost in all the concentrations). From the results minimum inhibitory concentrations of the leaf extracts were also determined and it was found to be 200 µg/ml; 300 µg/ml; 550µg/ml (Table: 4.11) respectively *against F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP) [(plate 8: fig. b; plate 9: fig. c and d; plate 10: fig. c and d)].

Table4.10: Inhibition of growth of three pathogens by different concentrations of leaf extract of *Datrura stramonium* (following agar cup method).

Plant extract	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg /ml)	Inhibition zone diameter (mm)	Extract Concentration (mg /ml)	Inhibition zone diameter (mm)
Aqueous leaf extract of: *Datura stramonium*	3.00	12±1.10	3.50	11±0.95	8.00	17±0.93
	2.00	11±1.54	3.00	09±0.78	7.00	14±1.15
	1.00	08±0.53	2.50	08±0.57	6.00	11±1.07
	0.50	07±0.15	2.00	07±0.06	5.00	08±1.73
	0.25	00	4.00	00	4.00	00
	control	00	control	00	control	00
CD5%		1.12		0.23		0.22

Data are mean of three replications

Table4.11: Inhibition of growth of three pathogens by different concentrations of bioactive fraction of leaf extract of *Datura stramonium* (following agar cup method).

Plant extract	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
	Extract Concentration (µg/ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)
bioactive fraction of leaf extract of: *Datura stramonium*	500	13±1.41	600	17±1.18	1000	14±0.99
	400	12±0.70	500	15±0.70	750	12±1.55
	300	11±1.31	400	13±0.87	600	11±0.47
	200	09±0.95	300	09±1.00	550	09±1.00
	100	00	200	00	500	00
	Control	00	Control	00	Control	00
CD5%		1.05		0.53		1.21

Data are mean of three replications

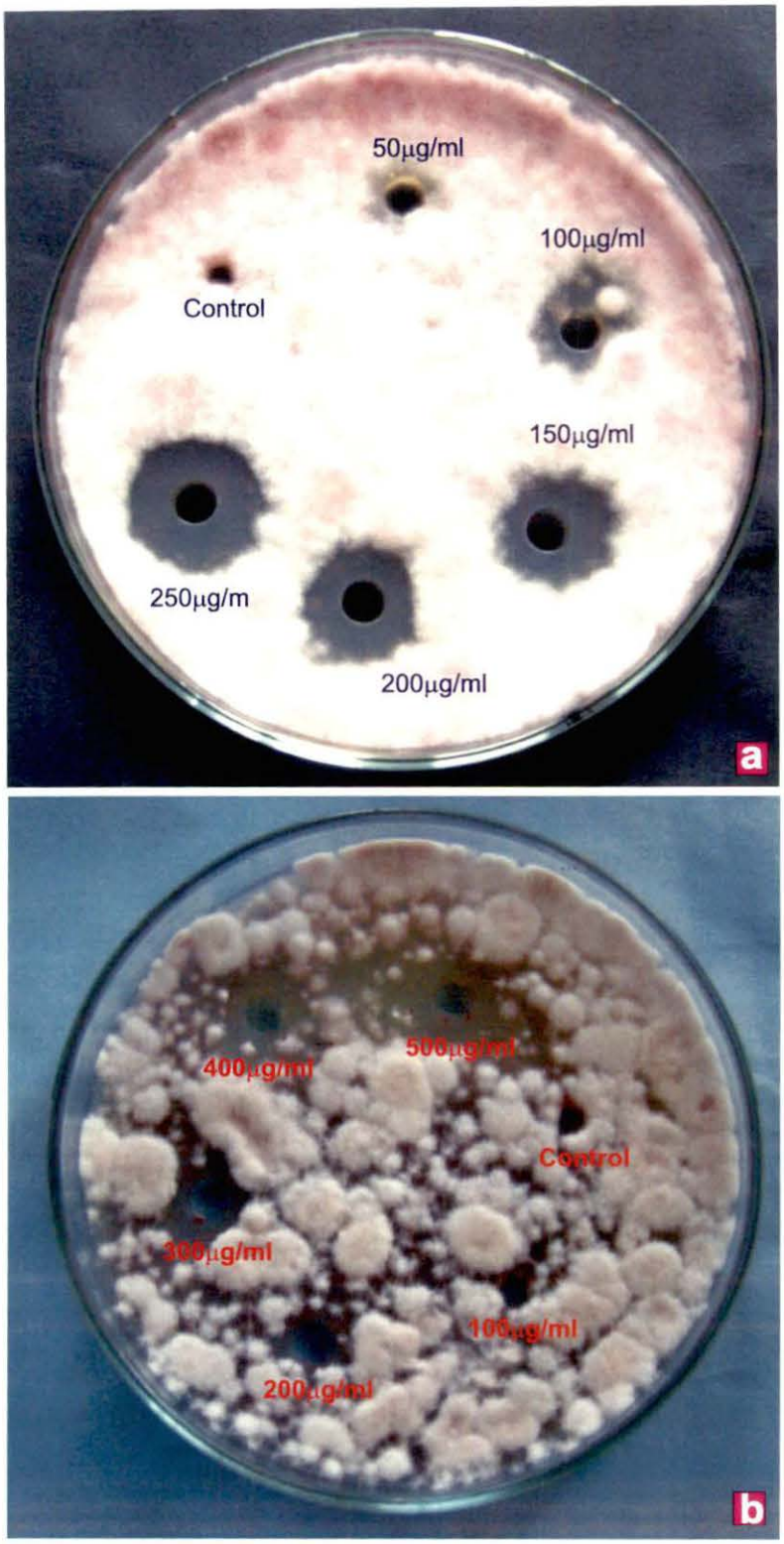


Plate 8:fig.a: Assessment of antifungal activity of bioactive fraction of *Xanthium strumarium* leaf extract against *Fusarium moniliforme*

fig.b: Assessment of antifungal activity of bioactive fraction of *Datura stramonium* leaf extract against *F. moniliforme.*

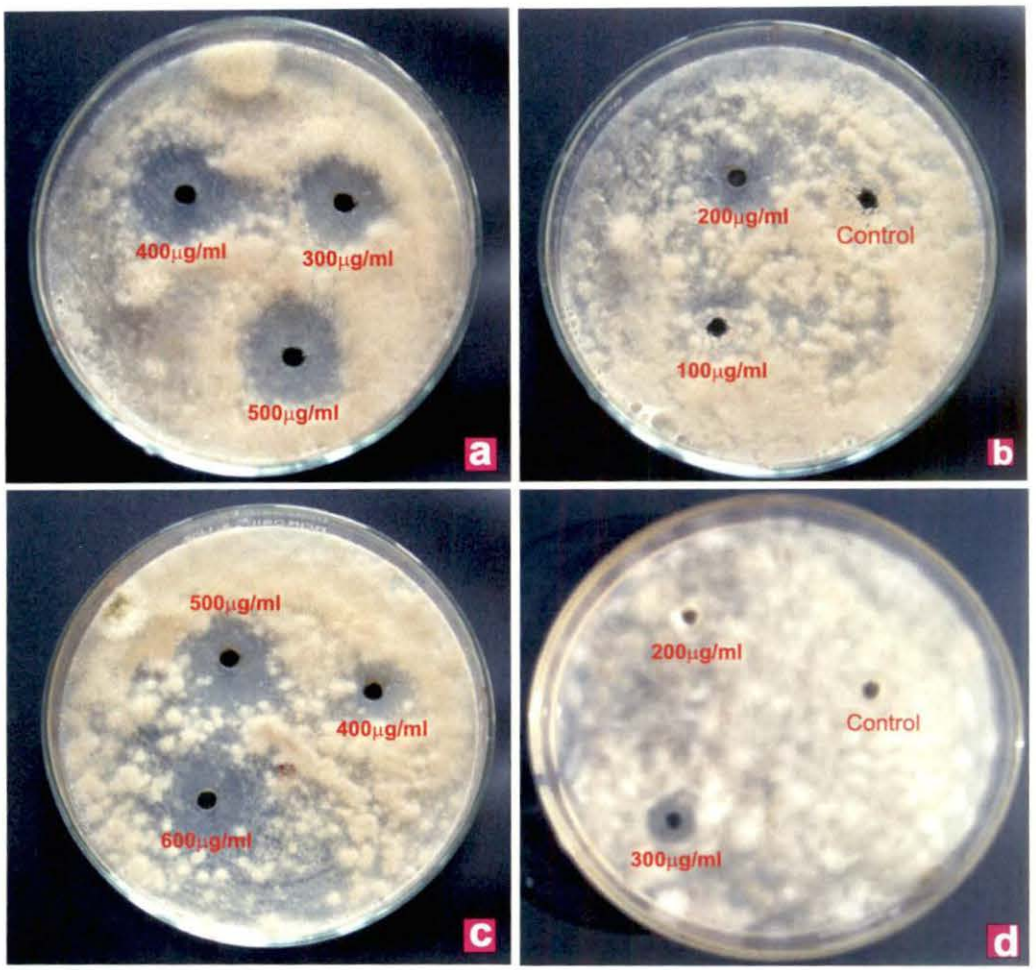


Plate 9: fig.a&b: Assessment of antifungal activity of bioactive fraction of *Xanthium strumarium* leaf extract against *Alternaria alternata* (AaT)

fig.c&d: Assessment of antifungal activity of bioactive fraction of *Datura stramonium* leaf extract against *Alternaria alternata* (AaT).

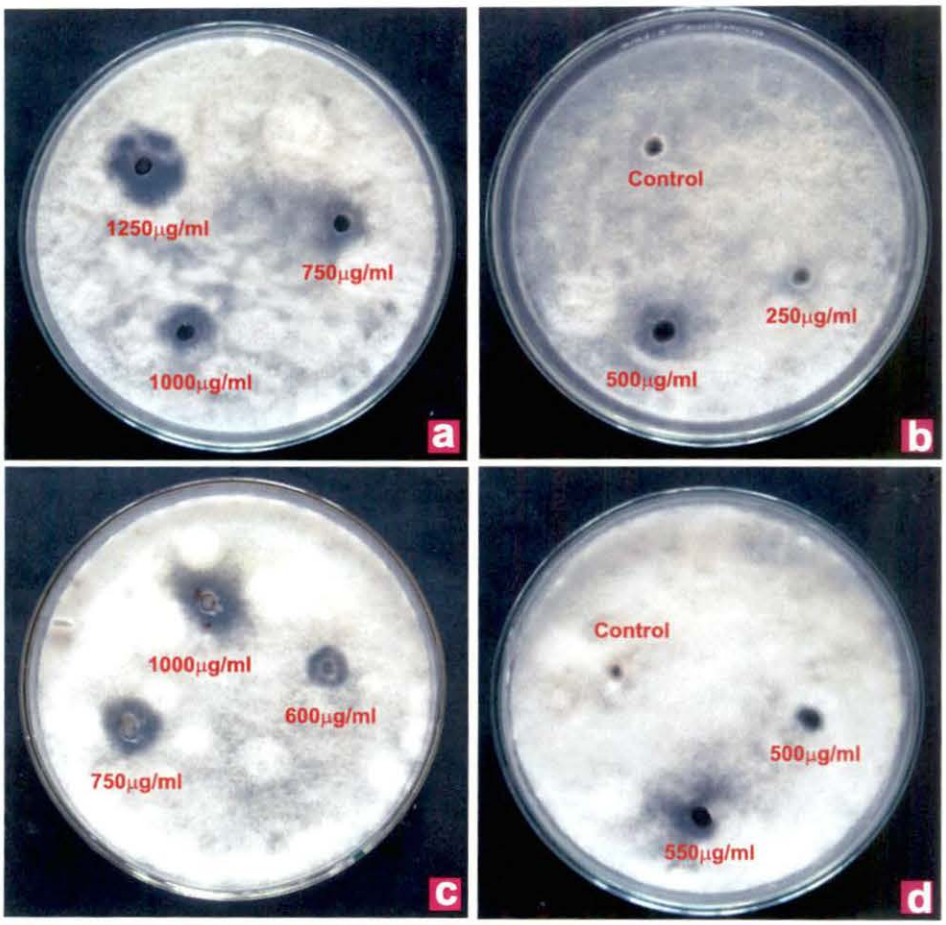


Plate 10: fig.a&b: Assessment of antifungal activity of bioactive fraction of *Xanthium strumarium* leaf extract against *Alternaria alternata* (AaP)

fig.c&d: Assessment of antifungal activity of bioactive fraction of *Datura stramonium* leaf extract against *Alternaria alternata* (AaP)

4.5: Fungicide sensitivity test.

Two commonly used fungicides were tested for their efficacy against the three fungal pathogens. Bioassay was done taking five different concentrations of two different fungicides 'Bavistin' and 'Roko'. It was also considered worthwhile to determine the MIC value of the fungicides tested against the three pathogens and to compare with the botanicals found to be potential in the present study. The five different concentrations were selected on the basis of their activity for determination of minimum inhibitory concentrations of the fungicides against the three selected fungi. Minimum inhibitory concentrations (MIC) were measured following standard techniques (Suleman *et al.* 2002; Saha *et al.* 2005a) as described in the materials and methods (section 3.5.8). Minimum inhibitory concentrations (MIC) were also determined against the three selected fungal pathogens of the present study. All the three [*Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)] fungal pathogens showed growth inhibition zones around agar cups containing effective concentrations of the fungicides. The lowest concentration which could inhibit growth of a fungi, were considered as MIC of that fungicide against the fungi tested.

Different concentrations of the fungicides were prepared by serial dilution of the fungicide solutions. Separate PDA plates, after solidification, were inoculated with 1 ml of spore suspension of *Fusarium moniliforme or Alternaria alternata* (AaT) or *Alternaria alternata* (AaP). Spore suspension was spread uniformly on the agar surface by a glass spreader. Three to four agar cups or wells (of 5 mm diameter) were made on the inoculated plates aseptically and 50 µl fungicides of different concentrations were poured in the wells. The wells were marked and the plates were incubated at 28 $\pm 1^0$ C in an incubator. The plates were observed after four days of inoculation and the least concentration in which the pathogenic fungi could not grow were considered as minimum inhibitory concentration. The results of agar cup bioassay against fungal pathogens have been presented in table-4.12 and table-4.13.

Five different concentrations of fungicide (Bavistin) were assessed for their efficacy against fungal pathogen *Fusarium moniliforme.* The test concentrations were 0.25, 0.50, 1.00, 1.50 and 2.00 mg/ml. Bavistin (1.00 mg/ml) was the minimum concentration to inhibit growth of the fungi (Table: 4.12; plate 11: fig. a).

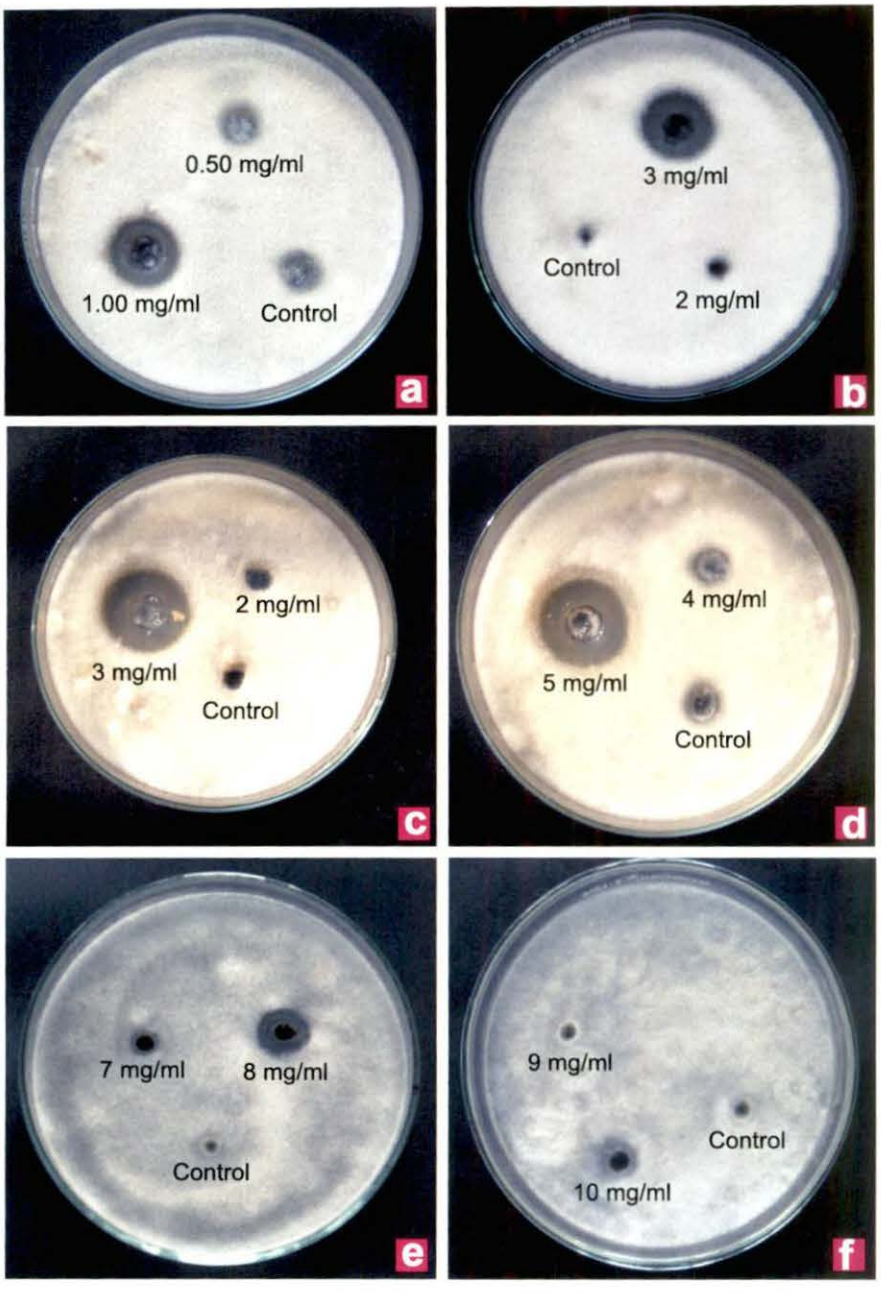


Plate 11: fig.a: Assessment of antifungal activity of Bavistin against *Fusarium moniliforme*

fig. b: Assessment of antifungal activity of Roko against *F. moniliforme*

fig. c: Assessment of antifungal activity of Bavistin against *Alternaria alternata* (AaT)

fig. d: Assessment of antifungal activity of Roko against *A. alternata* (AaT)

fig. e: Assessment of antifungal activity of Bavistin against *A. alternata* (AaP)

fig. f: Assessment of antifungal activity of Roko against *A. alternata* (AaP)

Table4.12: Inhibition of growth of the three fungal pathogens by different concentrations of Bavistin (following agar cup method).

Fungicide	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
Bavistin	Concentration (mg/ml)	Diameter of inhibition zone (mm)	Concentration (mg /ml)	Diameter of inhibition zone (mm)	Concentration (mg /ml)	Diameter of inhibition zone (mm)
	1.00	15±0.88	3.00	18±1.08	08.00	11±0.40
	0.50	00	2.00	00	07.00	00
	control	00	control	00	Control	00

Data are mean of three replications

Table4.13: Inhibition of growth of three fungal pathogens by different concentrations of Roko (following agar cup method).

Fungicide	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
Roko	Concentration (mg/ml)	Diameter of inhibition zone (mm)	Concentration (5g /ml)	Diameter of inhibition zone (mm)	Concentration (mg /ml)	Diameter of inhibition zone (mm)
	3.00	16±1.24	5.00	19±1.78	10.00	15±0.64
	2.00	00	4.00	00	9.00	00
	control	00	control	00	Control	00

Data are mean of three replications

In case of bioassay of Bavistin against the *Alternaria alternata* (isolate AaT) five test concentrations were 2.0, 3.0, 4.0, 5.0 and 6.0 mg/ml. Concentration 3.00 mg/ml was the minimum to inhibit the growth of the fungi (Table: 12; plate 11: fig. c). Five different concentrations of Bavistin (2.0, 4.0, 6.0, 8.0 and 10.0 mg/ml) were tested against another pathogen *Alternaria alternata* (isolate AaP) and MIC value of Bavistin against the fungus was found to be 8.00 mg/ml (Table 12; plate 11: fig e).

Similarly, fungicide roko was also used to control the three pathogens and were tested in the same concentrations as stated in case of bavistin. Bioassay studies ofRoko (Table 4.13) against the *Fusarium moniliforme, Alternaria alternata* (isolate AaT) *and Alternaria alternata* (isolate AaP) showed minimum inhibitory concentrations were higher than Bavistin. MIC value ofRoko was 3.00 mg/ml against *F. moniliforme* (Table 13; plate 11: fig. b). MIC of

Roko against the two *Alternaria alternata* isolates (isolate AaT and isolate AaP) was 5.00 mg/ml (Table: 13; plate 11: fig. d).and 10.00 mg/ml (Table: 13; plate 11: fig. f) respectively.

From the results it was evident that both the fungicides (Bavistin and Roko) were effective but Bavistin was better than theRoko as the minimum inhibitory concentration ofRoko was higher than that of Bavistin. If the fungicides are compared with the two plant extracts, it is clear that the leaf extracts (*Xanthium strumarium and Datura stramonium*) are more effective.

4.6: Agar cup method for detection of anti-bacterial activity by *Xanthium strumarium* & *Datura stramonium* and determination of MIC

Xanthium strumarium and *Datura stramonium* leaf extracts were tested following agar cup bioassay technique (section: 3.5.7) to test their antibacterial activity as well as to determine the minimum inhibitory concentrations. Radial diameters of inhibition zones, surrounding the agar cup, containing different concentration of leaf extracts, were recorded. MIC value was determined from the results of agar cup bio-assay. Aqueous and bioactive fraction of selected plant leaf extracts were tested for their efficacy against the three pathogens. Results have been presented in the table (4.14) to table (4.17). Each plant leaf extract was tested (against *Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp.).

Different concentrations of the botanicals were prepared by serial dilution of the 'aqueous leaf extracts' or 'bioactive fraction of leaf extracts' of two plants (*Xanthium strumarium* and *Datura stramonium*). One millilitre of bacterial suspension were mixed with 19 ml of NA media and finally plated in sterile petridish. After solidification three to four agar cups or wells (of 5 mm diameter) were made on the inoculated plates aseptically. 50 µl of aqueous leaf extracts or bioactive fraction of leaf extracts of the two plants (*Xanthium strumarium* and *Datura stramonium*) at different concentrations were poured in the wells. The wells were marked and the plates were incubated at 32 ± 1^0 C in an incubator. The plates were observed after 48 hours of inoculation and the least concentration in which the pathogenic bacteria could not grow were considered as minimum inhibitory concentration. The Results of agar cup bioassay against bacterial pathogens have been presented in table (4.14) to table (4.17).

Different concentrations (0.50, 0.75, 1.00, 1.50, 2.00 and 2.50 mg/ml), were made from the aqueous extract of *Xanthium strumarium.* All the three pathogenic bacteria were tested against the five different concentrations of *Xanthium strumarium* aqueous leaf extracts. The results of the tests have been presented in table (4.14). From the table it is evident that MIC

values of *Xanthium strumarium* (aqueous leaf extract) against *Xanthomonas* sp, *Pseudomonas syringae* and *Erwinia* sp. are 0.75mg/ml, 1.00mg/ml, and 1.00 mg/ml respectively.

Similarly, bioactive fraction of the *Xanthium strumarium* leaf extracts was also tested for their efficacy against the three bacteria *Xanthomonas* sp, *Pseudomonas syringae* and *Erwinia* sp. Details of the results have been presented in the table (4.15). From the tabulated values the minimum inhibitory concentrations were found to be 50µg/ml; 125µg/ml and 100µg/m (plate12: fig. a and b; plate13: fig. a and b; plate14: fig. a and b) respectively against the three isolated bacterial post harvest pathogens (*Xanthomonas* sp., *Pseudomonas syringae*. and *Erwinia* sp.).

Table 4.14: Inhibition of growth of three bacterial pathogens by different concentrations of leaf extracts of *Xanthium strumarium* (following agar cup method).

Plant extract	*Xanthomonas* sp.		*Pseudomonas syringae*		*Erwinia* sp.	
	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)
Aqueous leaf extract of: *Xanthium strumarium*	2.00	15±0.72	2.50	12±1.16	2.50	11±0.81
	1.50	13±0.85	2.00	10±1.65	2.00	09±1.19
	1.00	11±0.72	1.50	09±0.41	1.50	08±0.21
	0.75	09±0.72	1.00	07±0.41	1.00	07±0.21
	0.50	00	0.50	00	0.50	00
	control	00	control	00	Control	00
CD5%		0.42		0.18		0.35

Data are mean of three replications

Aqueous leaf extracts of *Datura stramonium* were also tested for determining effective inhibitory concentrations against the three bacteria. The original aqueous leaf extract was diluted to different concentrations (0.25, 0.50, 0.75, 1.00, 1.25, 1.50, 2.00 and 2.50 mg/ml) and tested against the three bacterial pathogens following agar cup bioassay. The details results have been presented in the table (4.16). MIC values of *Datura stramonium* (aqueous leaf extract) against *Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp *were* 0.50mg/ml; 1.00mg/ml; 0.75mg/ml respectively.

Table4.15: Inhibition of growth of three bacterial pathogens by different concentrations of bioactive fraction of leaf extracts of *X. strumarium* (following agar cup method).

Plant extract	*Xanthomonas* sp.		*Pseudomonas syrinage.*		*Erwinia* sp.	
	Extract Concentration (µg/ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)
Bio active fraction of leaf extract of: *Xanthium strumarium*	200	25±0.95	250	17±1.38	200	16±1.31
	150	22±1.55	200	15±0.40	150	14±0.55
	100	20±1.46	150	14±0.85	125	13±1.22
	50	14±0.82	125	13±0.20	100	12±0.68
	25	00	100	00	50	00
	control	00	Control	00	Control	00
CD5%		0.53		0.44		0.20

Data are mean of three replications

Finally, bioactive fraction of leaf extract of *Datura stramonium* was tested in seven different concentrations (25, 50, 75, 100, 125, 150, 200 and 250 µg/ml) for determining minimum inhibitory concentrations against *Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp. From the results (table 4.15) MIC values were found to be 50µg/ml; 100µg/ml and 75µg/ml respectively against the three bacteria (*Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp.; plate12: fig. c and d; plate13: fig. c and d; plate13: fig. c and d).

Table 4.16: Inhibition of growth of three pathogens by different concentrations of leaf extracts of *Datura stramonium* (following agar cup method).

Plant extract	*Xanthomonas* sp.		*Pseudomonas syringae*		*Erwinia* sp.	
	Extract Concentration (mg/ml)	Inhibition zone diameter (mm)	Extract Concentration (mg /ml)	Inhibition zone diameter (mm)	Extract Concentration (mg /ml)	Inhibition zone diameter (mm)
aqueous leaf extract of: *Datura stramonium*	2.00	13±0.62	2.50	14±0.78	2.00	10±1.32
	1.50	11±0.70	2.00	11±0.58	1.50	09±1.12
	1.00	08±0.32	1.50	10±0.67	1.00	08±0.88
	0.50	07±1.20	1.00	08±0.30	0.75	07±0.45
	0.25	00	0.50	00	0.50	00
	control	00	control	00	Control	00
CD5%		0.74		0.40		0.37

Data are mean of three replications

Table 4.17: Inhibition of growth of three pathogens by different concentrations of bioactive fraction of leaf extracts of *Datura stramonium* (following agar cup method).

Plant extract	*Xanthomonas* sp.		*Pseudomonas syringae*		*Erwinia* sp.	
	Extract Concentration (µg/ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)	Extract Concentration (µg /ml)	Inhibition zone diameter (mm)
Bioactive fraction of leaf extract of: *Datura stramonium*	200	18±0.79	200	19±0.78	250	18±0.87
	150	17±0.83	150	16±0.67	200	17±0.90
	100	14±0.70	125	14±0.62	100	13±0.82
	50	12±0.72	100	13±0.78	75	11±0.57
	25	00	50	00	50	00
	control	00	control	00	Control	00
CD5%		0.29		0.26		0.42

Data are mean of three replications

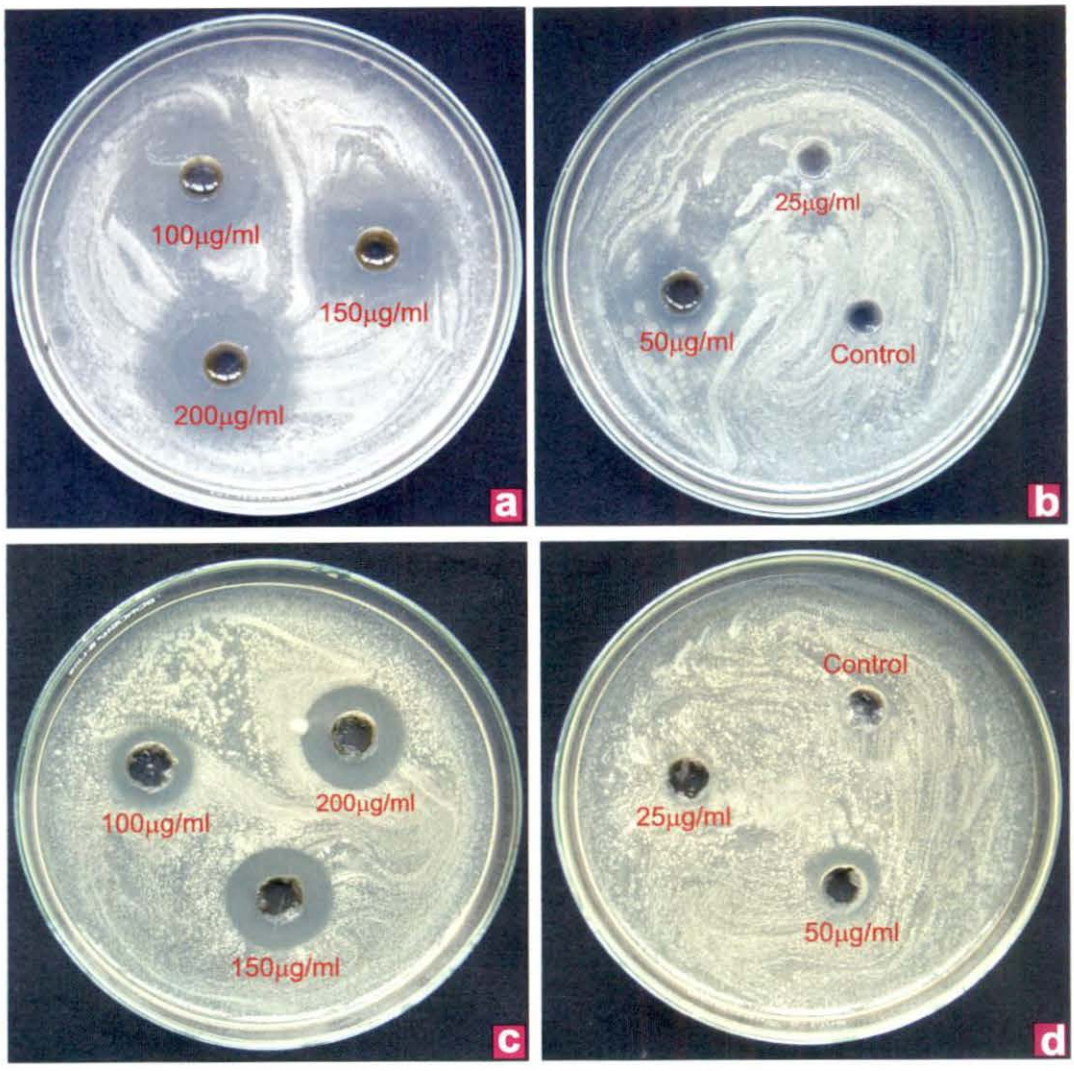


Plate 12: fig.a&b: Assessment of antibacterial activity of bioactive fraction of *Xanthium strumarium* leaf extract against *Xanthomonas* sp.
fig.c&d: Assessment of antibacterial activity of bioactive fraction of *Datura stramonium* leaf extract against *Xanthomonas* sp.

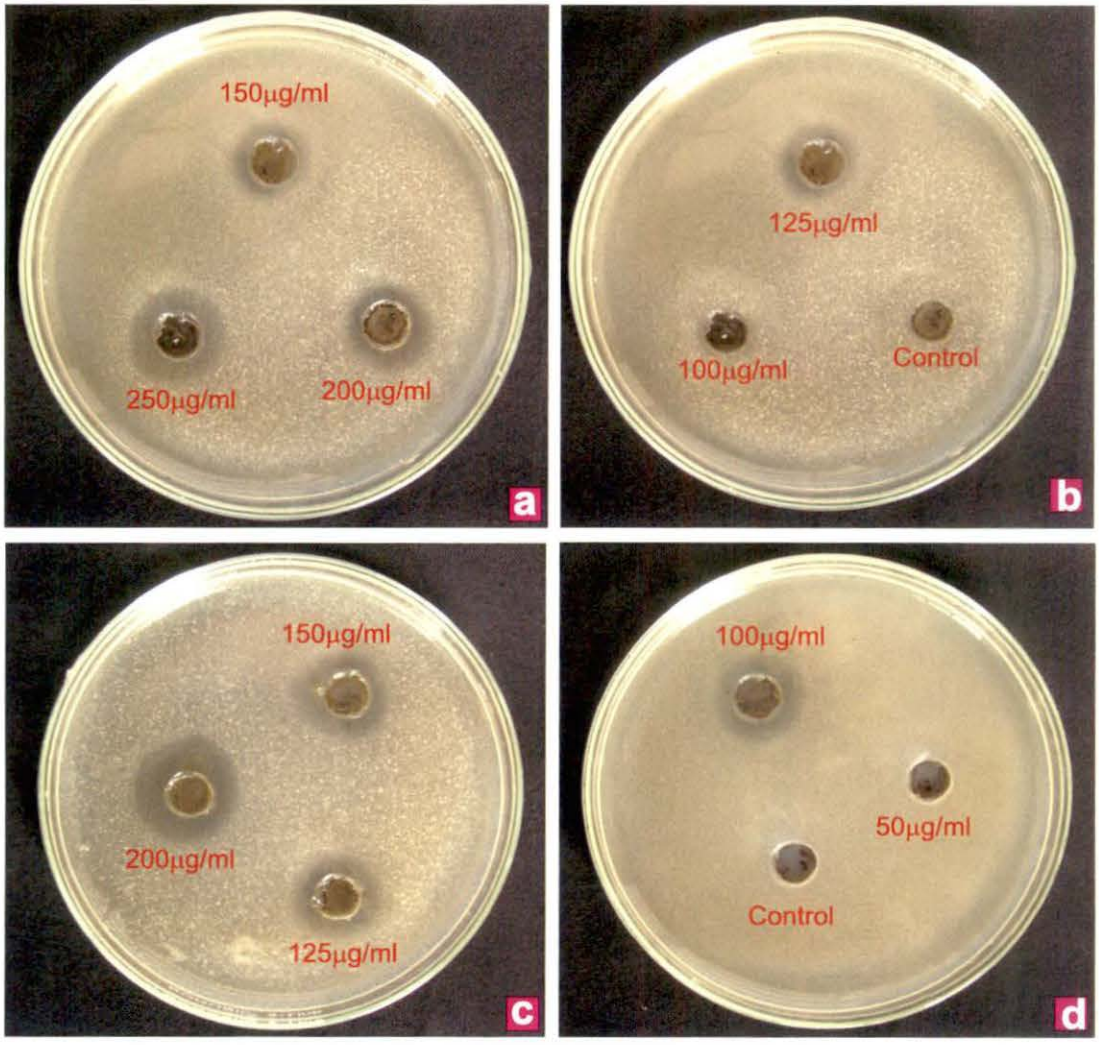


Plate 13: fig.a&b: Assessment of antibacterial activity of bioactive fractionl *Xanthium strumarium* eaf extract against *Pseudomonas syringae*.
fig.c&d: Assessment of antibacterial activity of bioactive fraction *Datura stramonium* leaf extract against *Pseudomonas syringae*.

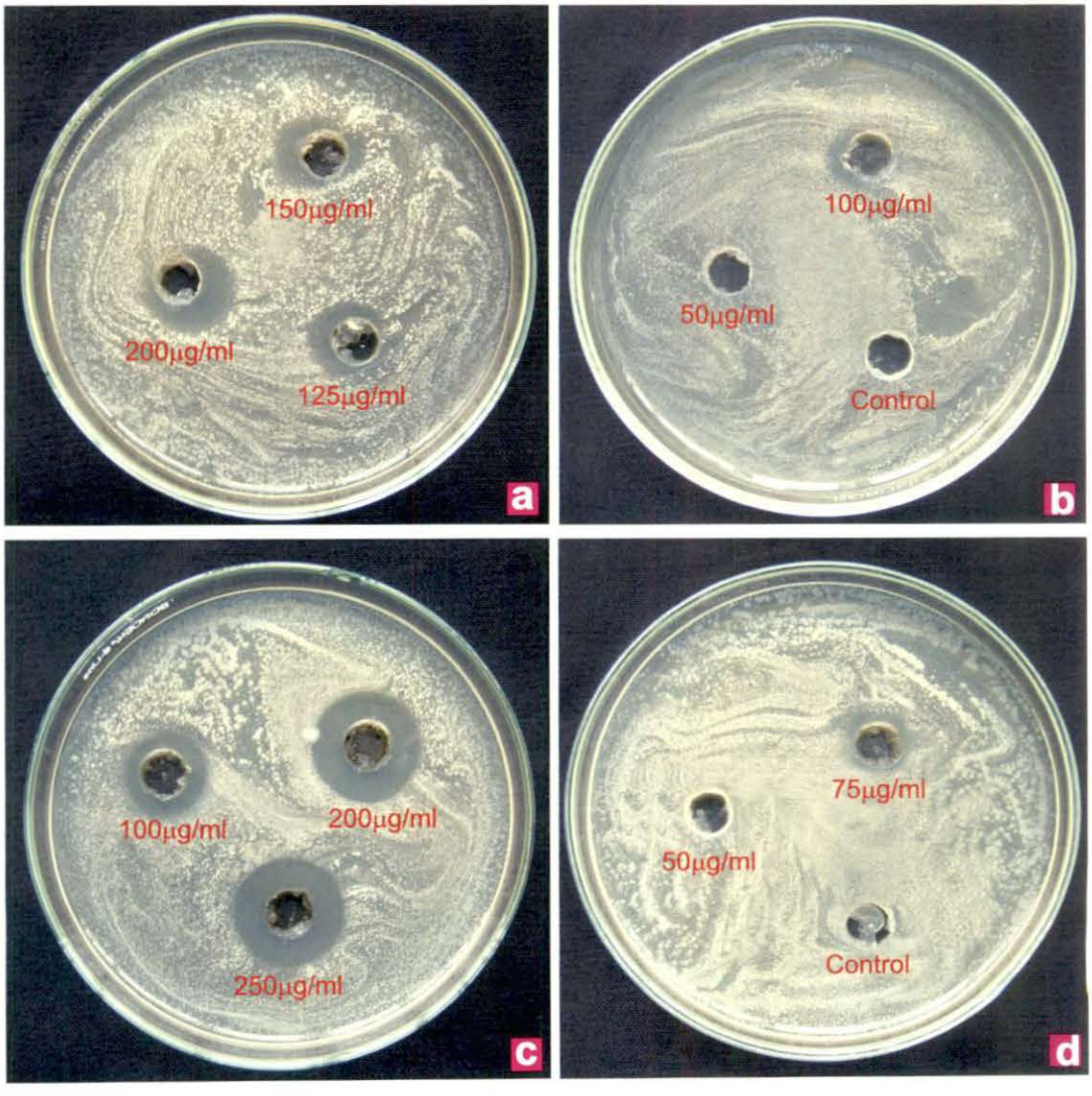


Plate 14: fig.a&b: Assessment of antibacterial activity of bioactive fraction of *Xanthium strumarium* leaf extract against *Erwinia* sp.
fig.c&d: Assessment of antibacterial activity of bioactive fraction of *Datura stramonium* leaf extract against *Erwinia* sp.

4.7: TLC plate bioassay for detection of antifungal properties of selected plant extracts.

It was evident from the results tabulated in table (4.6) that leaf extracts of *Xanthium srumarium* and *Datura stramonium* were effective against *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP). Therefore these extracts were selected for bioassay on thin layer chromatograms to detect presence of specific antifungal components which would produce inhibition zones at specific locations (R_f). Accordingly, these extracts were spotted on pre-activated TLC plates, developed in a solvent and dried and bioassay was done following procedures as described in materials and methods (Section: 3.5.9). After completion of the experiments, the R_f values of the inhibition zones were recorded and zone diameters were measured (Table: 4.18 and 4.21).

The selected plant leaf extracts (*Xanthium strumarium* and *Datura stramonium*; aqueous and bioactive fraction) were loaded on previously activated TLC plates and the plates were developed in two different solvent systems (mixtures) separately. The solvent systems along with the proportions of the different solvents have been given in the following lines. **Solvent system I=** chloroform: methanol:: 9:1 or **Solvent system II=** hexane: ethyl acetate : methanol :: 60:40:1 respectively. Spores of *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP) were mixed with Richards solution and sprayed on the developed plates by an automizer separately. The plates were placed in humid chambers separately at 28 ± 1^0C for 2-3 days. Details of the techniques have been presented in materials and methods, section: 3.5.9.

After 3 days of incubation the antifungal inhibition zones were observed and the diameter of the inhibition zones was measured. The R_f of the antifungal inhibition zones were also calculated and have been presented in table (18) to table (21). The plates showed distinct antifungal zones.

From the table (4.18) it is clear that R_f of antifungal zones created by the aqueous *Xanthium strumarium* leaf extracts (separated by solvent I on TLC plates) was 0.68 against both *Fusarium moniliforme* and *Alternaria alternata* (isolate AaP). The same

TLC plates when sprayed with *Alternaria alternata* (AaT) three antifungal zones (Zone 1: R_f 0.17, Zone 2: R_f 0.68 and Zone 3: R_f 0.82) were found. Similarly, partially purified bioactive fraction of *Xanthium strumarium* leaf extracts were developed in solvent-II and the developed plates were subjected to TLC plate bioassay against the three selected fungal pathogens. Antifungal zone at R_f-0.42 was found in case of all the three bioassays performed (plate 15: fig. a, plate 16: fig. a plate 17: fig. a). Two additional antifungal zones were found at R_f -0.17 and 0.72 in case of bioassay against *Alternaria alternata* (AaT) [plate 16: fig. a]. The diameter of the different zones were recorded and tabulated in the table (4.19).

Table 4.18: TLC plate bioassay of aqueous leaf extracts of *Xanthium strumarium against *Fusarium moniliforme, Alternaria alternata (AaT) and Alternaria alternata (AaP)*

No. of inhibition Zones	*Fusarium moniliforme*		*Alternaria alternata* (AaT)		*Alternaria alternata (AaP)*	
	Diameter of Inhibition zones (mm)	R_f	Diameter of inhibition zones (mm)	Rf	Diameter of Inhibition zones (mm)	R_f
Zone 1	--	--	15±0.40	0.17	--	--
Zone 2	17±0.76	0.68	16±0.78	0.68	17±1.05	0.68
Zone 3	--	--	15±0.90	0.82	--	--
CD 5%			0·61			

*Crude leaf extract 100 µg/ml. Data are mean of three replications
TLC plates developed in solvent system I = choloroform : methanol ::9:1

Table 4.19: TLC plate bioassay of bioactive fraction of *Xanthium strumarium leaf extract against *F. moniliforme, A. alternata (AaT) and A. alternata (AaP)*

No. of inhibition Zones	*Fusarium moniliforme*		*Alternaria alternata* (AaT)		*Alternaria alternata (AaP)*	
	Diameter of Inhibition zones	R_f	Diameter of inhibition zones	Rf	Diameter of Inhibition zones mm	R_f
Zone 1	--	--	14 ± 0·58	0.17	--	--
Zone 2	27±0.87	0.42	18±0.64	0.42	19±0.38	0.42
Zone 3	--	--	15 ± 0·46	0.72	--	--
CD 5%			0·16			

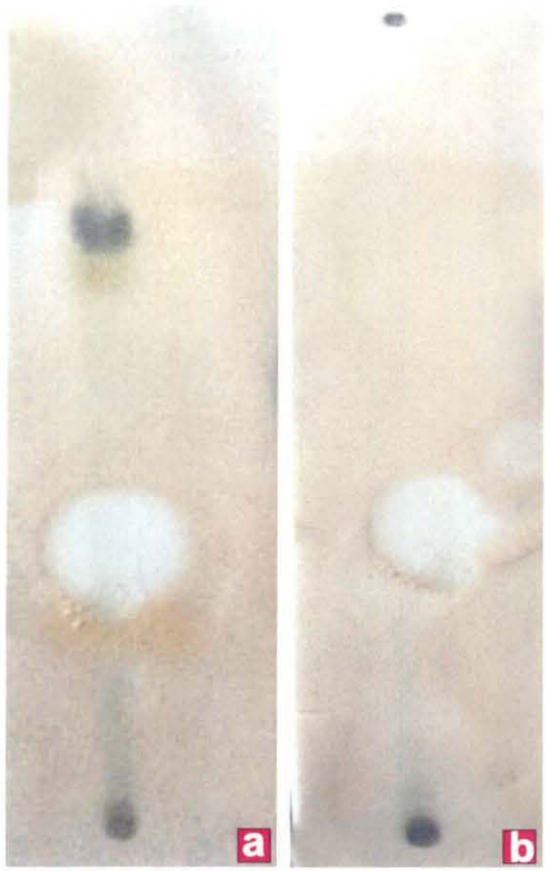


Plate 15: TLC plate Bioassay **fig. a:** Bioactive fraction of *Xanthium strumarium* leaf extract against *Fusarium moniliforme*
fig. b: Bioactive fraction of *Datura stramonium* leaf extract against *Fusarium moniliforme*

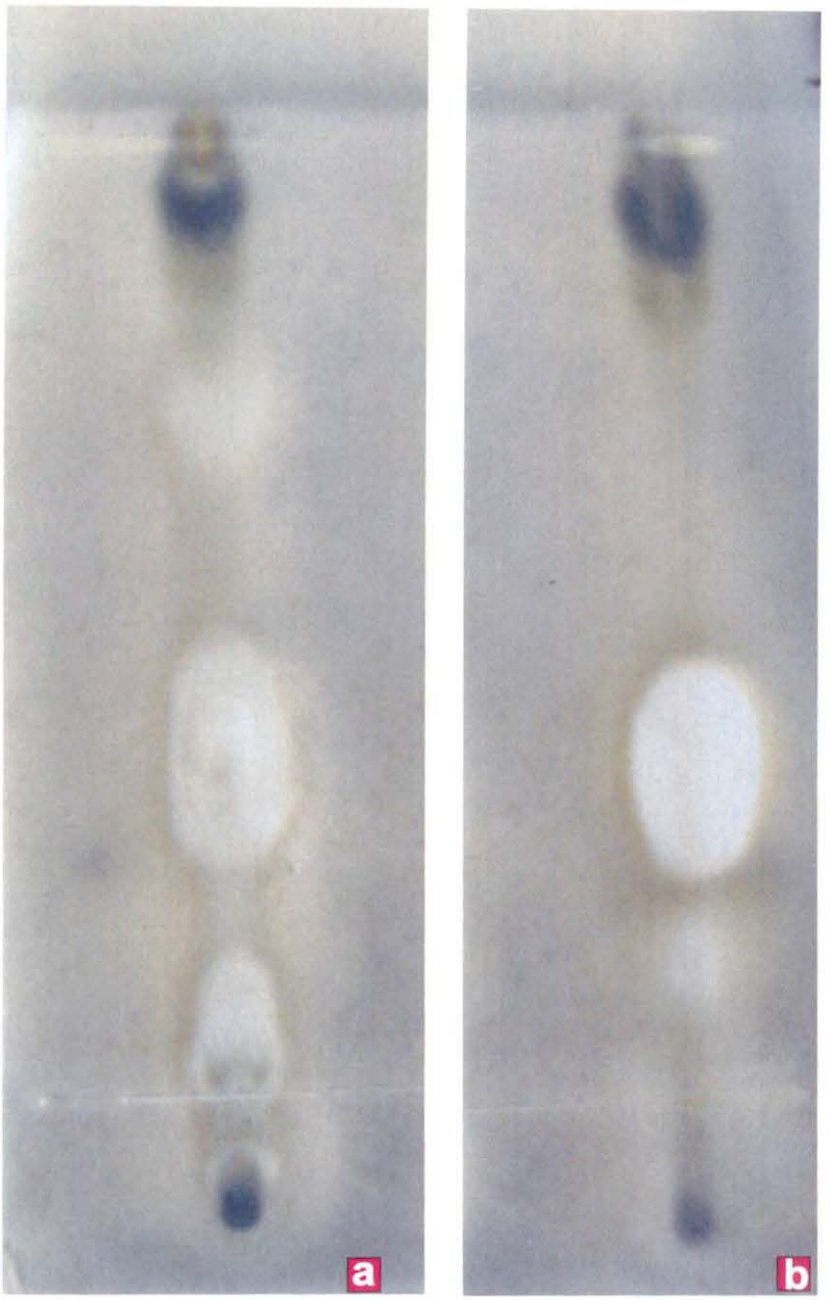


Plate 16: TLC plate Bioassay **fig. a:** Bioactive fraction of *Xanthium strumarium* leaf extract against *Alternaria alternata* (AaT)
fig. b: Bioactive fraction of *Datura stramonium* leaf extract against *Alternaria alternata* (AaT)

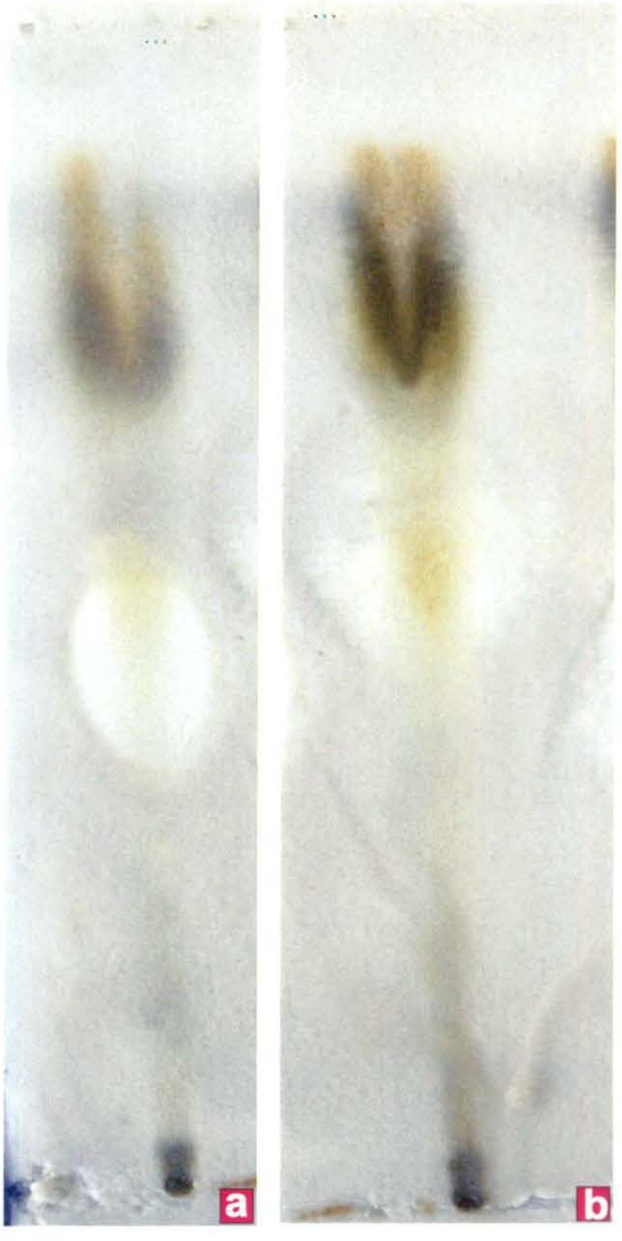


Plate 17: TLC plate Bioassay **fig. a:** Bioactive fraction of *Xanthium strumarium* leaf extract against *Alternaria alternata* (AaP)
fig. b: Bioactive fraction of *Datura stramonium* leaf extract against *Alternaria alternata* (AaP)

*Bioactive fraction of leaf extract 100 µg/ml. Data are mean of three replications. TLC plates developed in solvent system II= hexane: ethyl acetate : methanol::60:40:1

Similarly, from the table (4.20) it is clear that R_f of antifungal zones created by the aqueous *Datura stramonium* leaf extracts (separated by solvent I on TLC plates) *were* 0.63, 0.72 and 0.81 against *Alternaria alternata* (AaT) respectively. When the TLC plate sprayed with *Fusarium moniliforme* one antifungal zone was found at R_f- 0.72. Similarly, in case of bioassay where *Alternaria alternata* (AaP) were used, one antifungal zone was found at Rf- 0.81.

Table-4.20: TLC plate bioassay of aqueous leaf extracts of *Datura stramonium against *Fusarium moniliforme*, *Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)

No. of inhibition Zones	*Fusarium moniliforme*		*Alternaria alternata* (AaT)		*Alternaria alternata (AaP)*	
	Diameter of Inhibition zones	R_f	Diameter of inhibition zones	Rf	Diameter of Inhibition zones mm	R_f
Zone 1	--	--	12±0.45	0.63	--	--
Zone 2	17±0.95	0.72	18±0.87	0.72	--	--
Zone 3			14±0.56	0.81	14±0.53	0.68

*Aqueous leaf extracts 100µg/ml. TLC plates developed in solvent choloroform : methanol ::9:1

Finally, partially purified bioactive fraction of *Datura stramonium* leaf extracts were developed in solvent-II and the developed plates were subjected to TLC plate bioassay against the three selected fungal pathogens separately. Altogether three antifungal zones were found in the bioassays. One antifungal zone (R_f-0.37; plate 15: b) was found against *Fusarium moniliforme* which was not found in other two cases. Two antifungal zones (Rf- 0.20 & 0.47; plate 16: b). were found against *Alternaria alternata* (AaT) but the other isolate of *Alternaria alternata* (AaP) showed antifungal zone at R_f- 0.47 only (plate 17: b). The diameter of the antifungal zones was recorded in the table (4.21).

Table-4.21: TLC plate bioassay of bioactive fraction of **Datura stramonium* leaf extract *against F. moniliforme, A. alternata* (AaT) and *A. alternata* (AaP)

No. of inhibition Zones	*Fusarium moniliforme*		*Alternaria alternata* (AaT)		*Alternaria alternata (AaP)*	
	Diameter of Inhibition zones(mm)	R_f	Diameter of inhibition zones	Rf	Diameter of Inhibition zones mm	R_f
Zone 1	-	-	06±0.50	0.20	-	-
Zone 2	15.3±0.36	0.37	-	-	-	-
Zone 3	-	-	20±0.62	0.47	23±0.52	0.47

*Bioactive fraction of leaf extract 100 µg/ml. TLC plates developed in solvent hexane: ethyl acetate : methanol::60:40:1

4.8: Study of chromatograms following spray of chromogenic reagents

Antifungal zones on the TLC plates (showed by bioactive fraction of *Xanthium* leaf extract) were compared with a fresh TLC plate developed in the same solvent and sprayed with Vanillin-H2SO4. From the results (Plate 18: fig. a) it is evident that the one zone (Zone-1, R_f -0.42) were chromogenic spray (vanillin-H_2SO_4) positive and shows the indication of the antifungal constituent as terpenoid / phenolic compounds.

Similarly, Antifungal zones on the TLC plates (showed by bioactive fraction of *Datura* leaf extract) were compared with a fresh TLC plate developed in the same solvent and sprayed with Vanillin-H_2SO_4. From the results (Plate 18: fig b) it is evident that the two zones (Zone-2= R_f– 0.37 & zone-3= R_f -0.47) were chromogenic spray positive and showed the indication of the antifungal constituent as terpenoids/ phenolic compounds. However the zone-1 was Vanillin- H_2SO_4 spray reagent negative.

4.9: Structural identification of the antifungal compound

The purity of the fraction from the extract of *Xanthium strumarium,* which showed potential antifungal activity, was checked on TLC (Merck aluminum plates coated with silica gel 60, F_{254}) with R_f = 0.42 (TLC run in a mixture of solvents: hexane: ethyl acetate: methanol in the ratios of 60:40:1) and developed by UV and spraying with chemical developer. The spectral analyses were conducted by UV-Vis (Shimadzu, Japan), IR (Shimadzu-8300, Japan) spectrophotometer and by ^{1}H- and ^{13}C-NMR (Bruker-

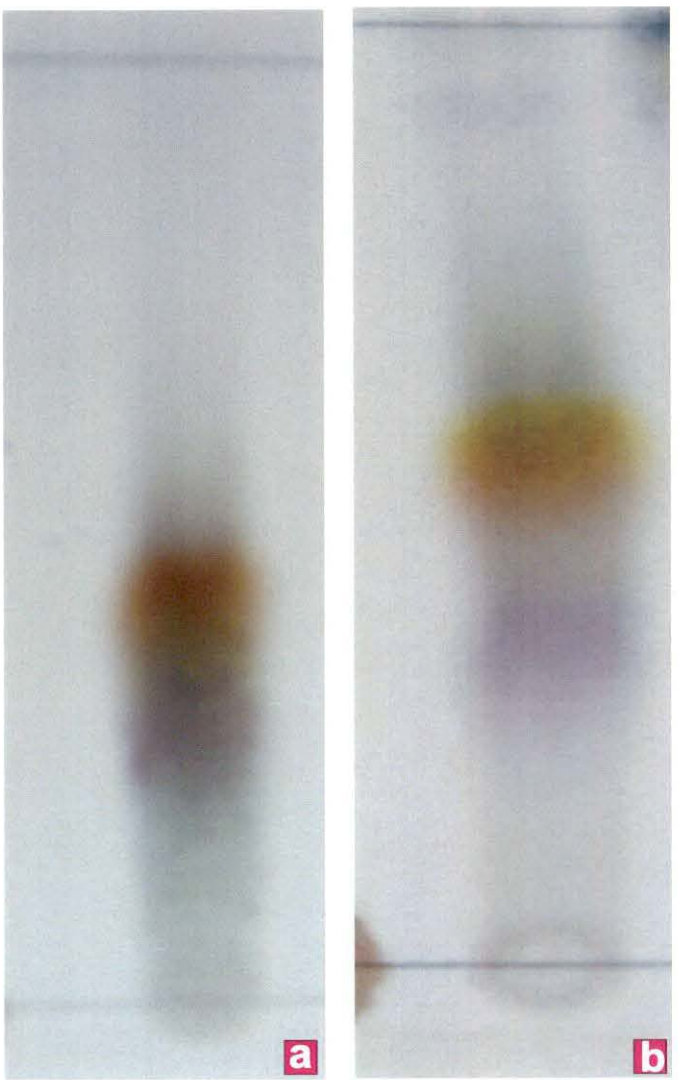


Plate 18: Identification of bioactive fraction on TLC plate assay by developer.
fig. a: *Xanthium strumarium* leaf extract
fig.b: *Datura stramonium* leaf extract

AV 300, Germany), operating respectively at 300 MHz and 75 MHz and using tetramethylsilane (TMS) as internal standard.

Spectral analyses:

The UV spectrum of the bio-active compound was taken in EtOH solution. A strong absorbance with λ_{max} was observed at 277.4 nm indicating presence of unsaturated carbonyl function. The infrared absorption spectrum was run in neat and ν_{max} were observed mainly at 1758, 1750, 1660 cm^{-1}, which revealed the presence of carbonyl groups (corresponding to keto and lactone carbonyl) and C=C double bond. The ^{1}H-NMR spectrum of the bio-active sample in $CDCl_3$ showed several characteristic peaks that led to assume that the compound may have structure **1**. For example, two peaks at δ 6.95 [d, J = 16 Hz, (approx.) calculated by scale measurements] and δ 6.15 [d, J = 16 Hz (approx.)] indicate two olefinic protons coupled to each other as *vicinal* coupling attached with C-2 and C-3 having *trans* configuration (as noticed from high value of coupling constant, J). On the other hand, two vinyl protons at C-13 appeared as doublets (*geminal* coupling) at δ 5.55 and δ 6.30 ppm with J = 2 Hz (approx.). These two vinylic protons attached at C-13 should have different chemical shifts because of the rigidity around the double bond and different electronic environments. Highly deshielded proton resonating at δ4.62 ppm (as a triplet) could be attributed to the methine proton attached with lactone oxygen at C-8. A sharp singlet appearing at δ 2.29 ppm indicates the presence of one methyl group (C-15) attached with carbonyl group (i.e. $COC\underline{H}_3$). Further presence of the methyl group was exhibited at δ1.18 ppm as a doublet (J = 7 Hz), which could be assigned to C-14 methyl group. The ^{13}C-NMR spectrum (fully decoupled) also corroborated the structure of the compound **1**. Thus, seven aliphatic carbons were displayed in the range of δ 21.5 – 36.5 ppm and six olefinic carbons appeared in the range of δ 122.6 – 149.5 ppm. The C-13 olefinic carbon appeared at δ 122.6 ppm, as also observed by Dong Kil. *et al.*, (2002) in their studies on structural identification of antifungal compound extracted from *Xanthium strumarium*. Unfortunately, because of low concentration of the bio-active antifungal compound, the carbonyl carbons (for the keto and lactone carbonyl groups) were difficult to recognize from noises. However, combination of all spectral data followed by analyses, as depicted above, led us to

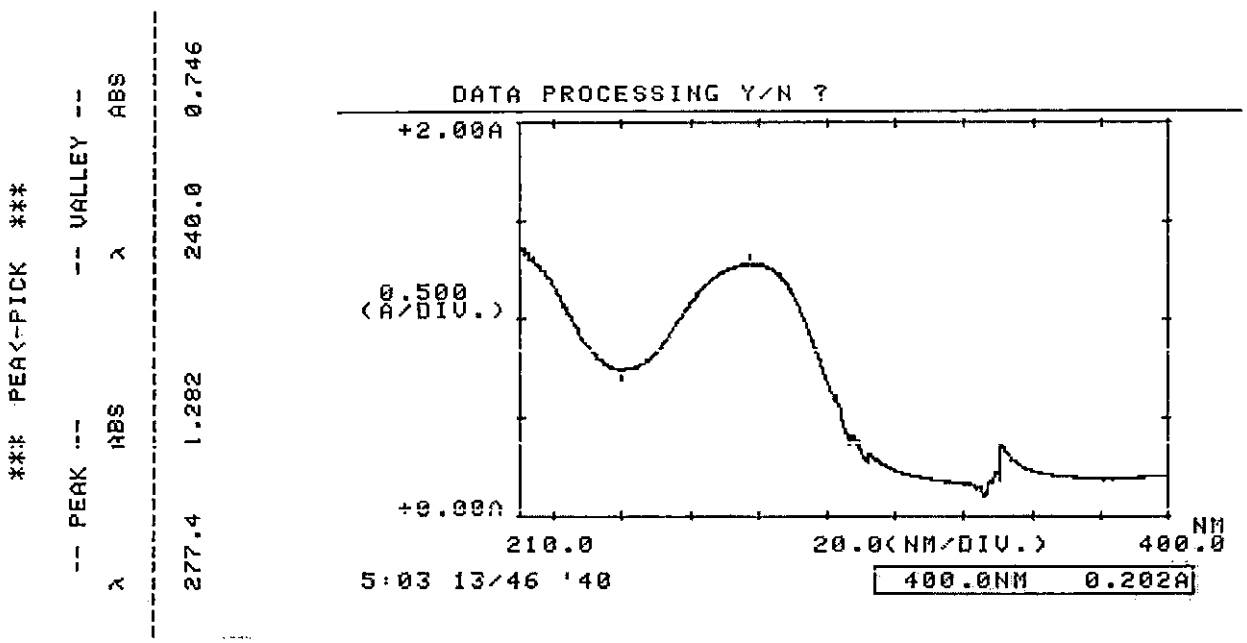


Fig: 3. Ultra violet absorption spectrum of the potential antifungal compound isolated from leaf extract of *Xanthium strumarium*. The solvent used 100% MeOH.

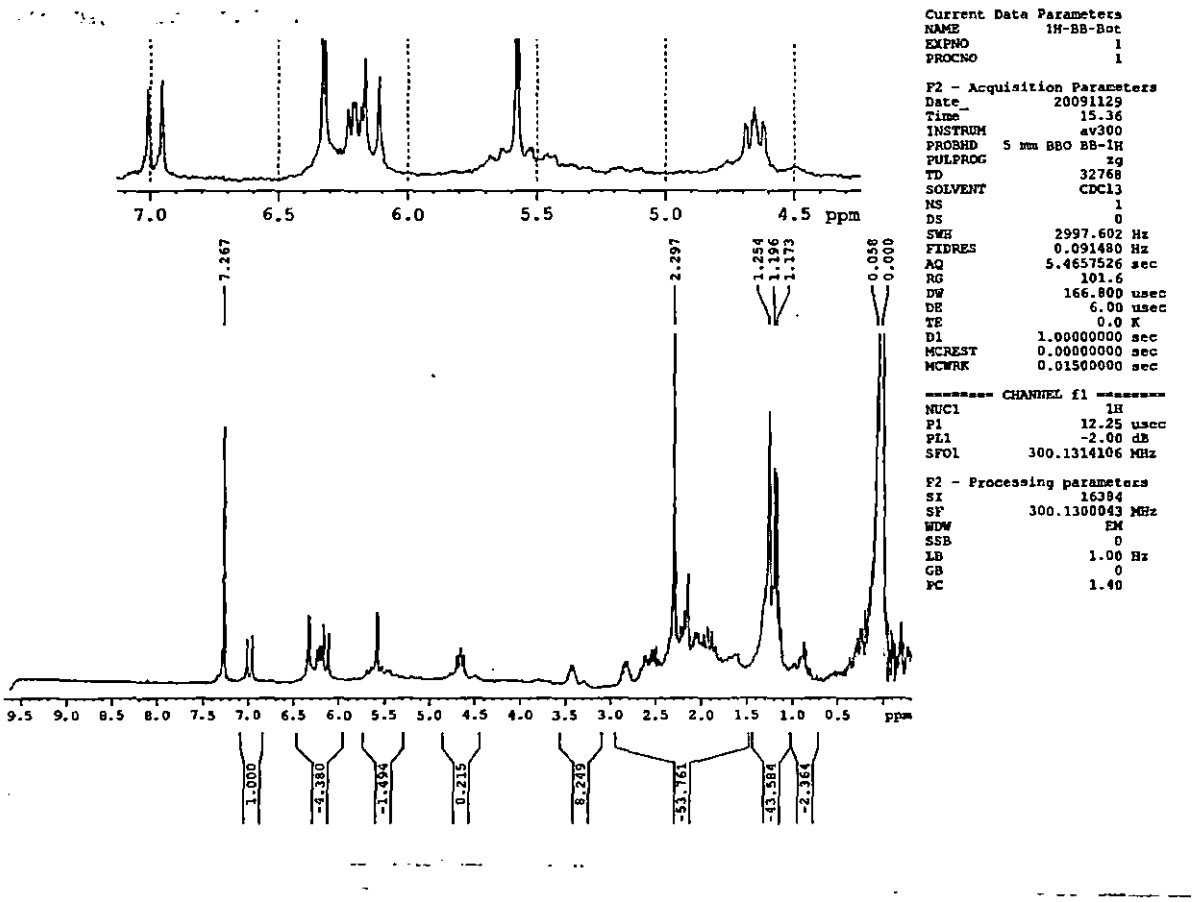


Fig: 4. ^{1}H-NMR spectrum of the potential antifungal compound isolated from leaf extract of *Xanthium strumarium.*

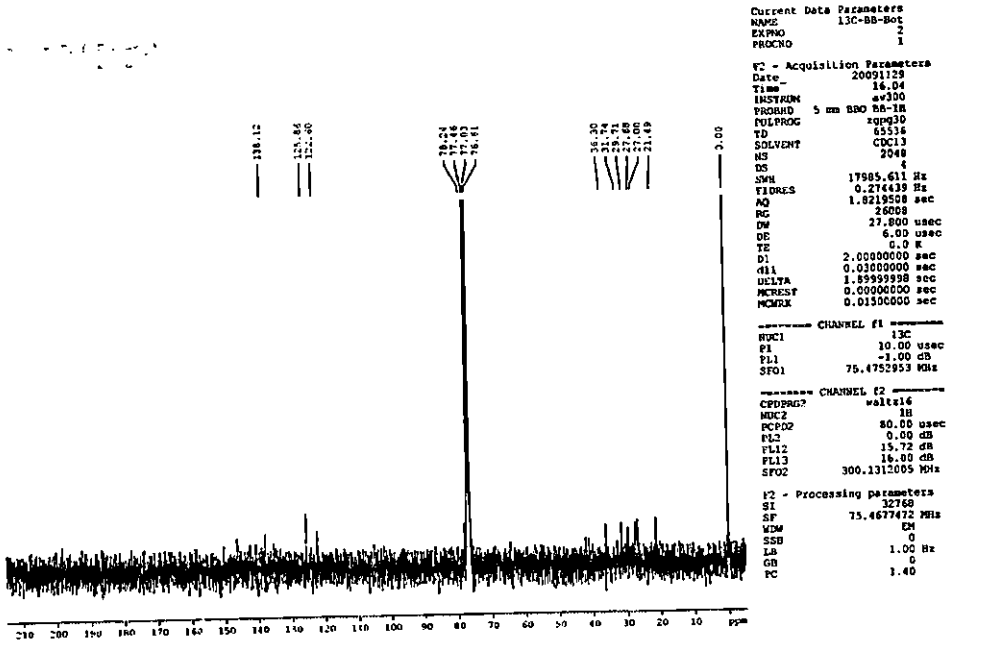


Fig: 5. ^{13}C-NMR analysis of the potential antifungal compound isolated from leaf extract of *Xanthium strumarium*.

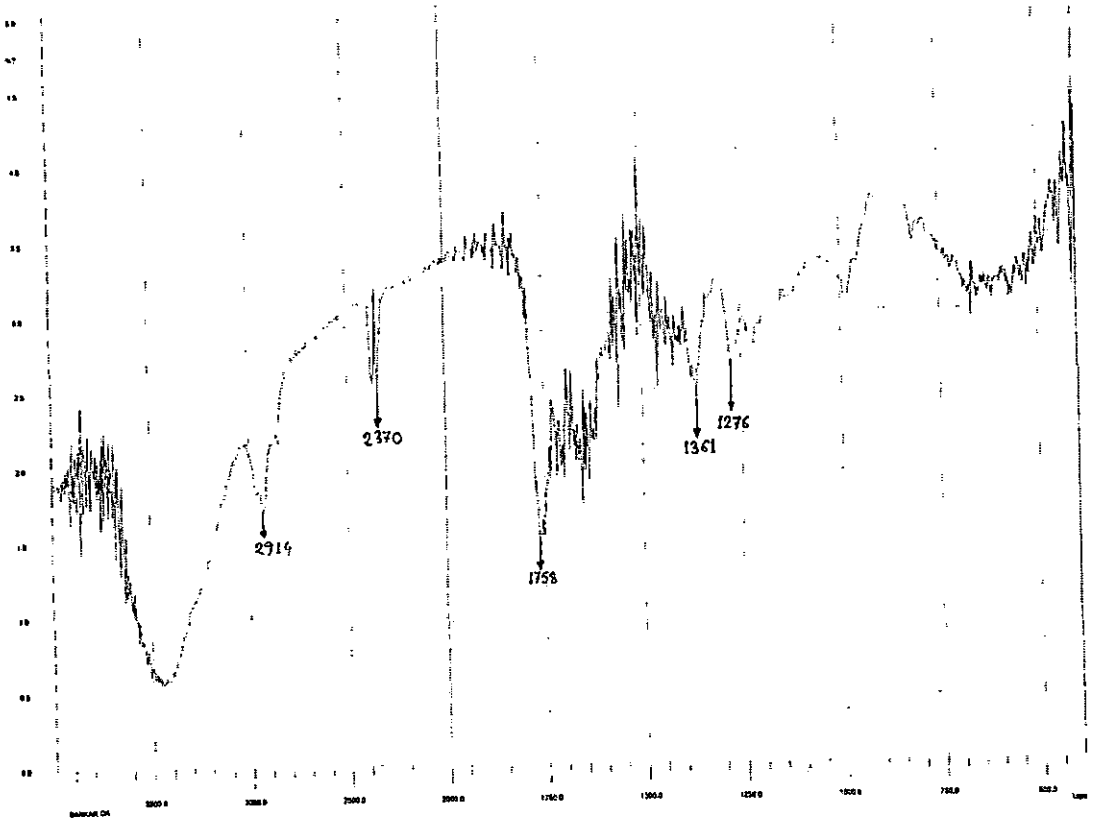
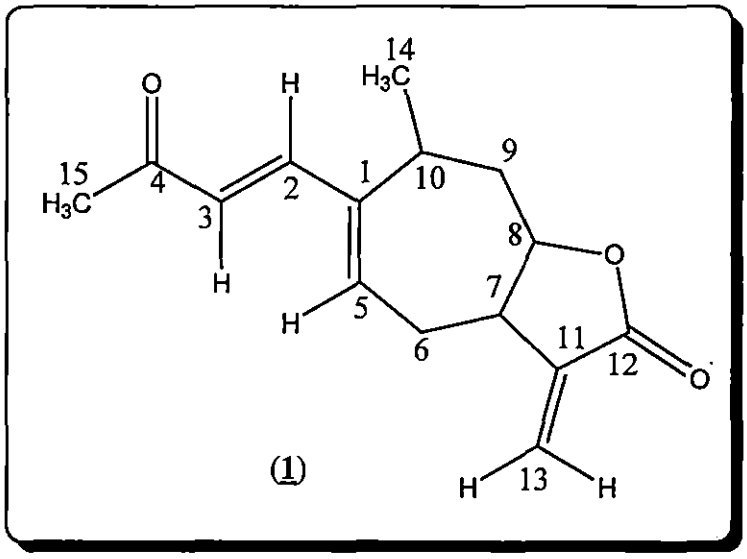


Fig: 6. Infra red spectra of the potential antifungal compound isolated from leaf extract of *Xanthium strumarium*.

tentatively propose that the structure of the bio-active component possessing antifungal activity may have the chemical structure 1. Further isolation, purification and spectral analyses would be undertaken in future studies to establish its structure in a more conclusive manner.

In summary, we have isolated and purified the extracts of *Xanthium strumarium*, which has shown potential antifungal activity towards vast array of pathogens. Furthermore, its chemical identity has been established through analyses of its spectral data. The proposed structure of the bio-active compound has been assigned chemical structure (1), which is also corroborated in the investigation made by Dong Kil *et al.* (2002).



(1)

Tentative structure of antifungal compound extracted from *Xanthium strumarium*

4.10: Application of botanicals on the three fruits and assessment of disease.

4.10.1: Application of botanicals and reduction of disease incidence in susceptible oranges.

Pathogenicity test of *F. moniliforme and Xanthomonas* sp. were performed on oranges collected from different places of North Bengal with a view to get most susceptible oranges for future experiments. This was necessary to get most susceptible oranges. Altogether 100 spots were made on the surface of ten oranges and inoculated with the test pathogen. The inoculation technique has been described in details in the materials and methods, section: 3.5.10. From the results (Table: 4.22) it is clear that all the oranges collected from four different places were susceptible. But oranges collected

from Garubathan were the most susceptible. Hence these oranges were selected for post harvest disease control experiments following application of botanicals. *In vivo* application of botanicals and assessment of disease incidence in the fruits following challenge inoculation by the pathogens were performed. Results (Table: 4.23) revealed that disease caused by *F. moniliforme* was reduced 93.13% by the application of the bioactive fraction of the *Xanthium* leaf extract at a concentration of 100µg/ml. At same concentration, *Xanthium* leaf extract (bioactive fraction) reduced 92.32% disease, caused by and *Xanthomonas* sp.

Table 4.22: Pathogenicity of *F. moniliforme* and *Xanthomonas* sp. collected from different places of North Bengal

Orange collected from	Inoculated by *F. moniliforme*		Inoculated by *Xanthomonas* sp.	
	Percent lesion formed after 6 days	Total area of the damaged tissue (necrotic lesions) after 6 days* (cm^2)	Percent lesion formed after 6 days	Total area of the damaged tissue (necrotic lesions) after 6 days * (cm^2)
Mirik	56±0.97	117±1.39	73±0.96	138±1.55
Kalimpong	47±0.95	120±0.95	67±1.02	127±1.37
Garubathan	70±1.06	174±1.35	79±0.62	150±1.51
Lava	41±1.19	128±0.95	51±1.46	96±1.65
CD5%	0.61	0.72	0.97	0.64

*Cumulative data of 100 spots in 10 fruits. No lesion was found in control oranges

Table 4.23: Disease incidences (total area of necrotic lesions) following application of *Xanthium strumarium* leaf extracts in susceptible oranges collected from Garubathan area.

Inoculated by *F. moniliforme*			Inoculated by *Xanthomonas* sp.		
Total area of the damaged tissue (Necrotic lesions) after 6 days (cm^2)		Percent reduction in disease incidence	Total area of the damaged tissue (Necrotic lesions) after 6 days (cm^2)		Percent reduction in disease incidence
Untreated	*Pre-treated		Untreated	*Pre-treated	
174.8	12.0±0.00	93.13±0.00	150.1±0.00	10.7±0.00	92.32±0.00

*Pre-treated by spraying bioactive fraction of *Xanthium strumarium* leaf extract at a concentration of 100µg/ml.

4.10.2: Application of botanicals and reduction of disease incidence in susceptible tomatoes.

Pathogenicity test of *A. alternata* (isolate AaT) and *Pseudomonas syringae* were performed on tomatoes, collected from different tomato growing places of North Bengal. This was performed for getting most susceptible tomatoes. Altogether 100 spots were made on the surface of ten tomatoes and inoculated with the test pathogens. The inoculation technique has been described in details in the materials and methods section: 3.5.10. From the results (Table: 4.24) it is evident that the tomatoes collected from Haldibari were more susceptible.

Most susceptible oranges of Haldibari were pretreated by *Xanthium strumarium* leaf extract (bioactive fraction, concentration of 100µg/ml) and inoculated by the pathogens separately. Reduction in disease incidence was assessed in comparison to untreated-inoculated fruits. Results (Table: 4.24) revealed that disease (caused by *A. alternata,* isolate AaT) was reduced up to 88.33% by the application of the terpenoid fraction of the *Xanthium* leaf extract (at a concentration of 100µg/ml). The same experiment was also performed to control disease caused by *Pseudomonas syringae.* Disease incidence was reduced up to 91.7% in comparison to untreated-inoculated control set (Table: 4.25). There was no disease in the untreated-uninoculated control.

4.10.3: Application of botanicals and reduction of disease incidence in susceptible pineapples.

At the onset of this experiment pathogenicity of *A. alternata* (AaP) and *Erwinia* sp. were performed on pineapples collected from four different pineapple growing areas of North Bengal. This was done to get most susceptible pineapples. Altogether 100 spots were made on the surface of five pineapples and inoculated with the test pathogens. The inoculation technique has been described in details in the materials and methods section: 3.5.10. From the results (Table 4.26) it is clear that all the pineapples collected from four different places were susceptible. Pineapples collected from Bidhannagar were most susceptible.

Table 4.24: Pathogenicity of *A. alternata*(AaT) and *Pseudomonas syringae* on tomato collected from different places of North Bengal.

Tomato collected from	Inoculated by *A. alternata*(AaT)		Inoculated by *Pseudomonas syrinage*	
	Percent lesion formed after 6 days	Total area of the damaged tissue(necrotic lesions) after 6 days * (cm^2)	Percent lesion formed after 6 days	Total area of the damaged tissue (necrotic lesions) after 6 days * (cm^2)
Birpara	48±0.75	85.±1.47	56±1.05	106±1.96
Falakata	59±0.95	100±1.62	61±0.50	115±1.50
Haldibari	69±1.08	117±2.47	77±1.03	146±1.77
Phansidwa	49±1.23	83±1.23	43±1.00	81±1.45
CD 5%	0.75	1.84	0.80	2.18

* Data of 100 spots in 10 fruits. No lesion was found in control tomatoes.

Table 4.25: Disease incidences (total area of necrotic lesions) following application of *Xanthium strumarium* leaf extracts in susceptible tomatoes collected from Haldibari area.

Inoculated by *A. alternata* (AaT)			Inoculated by *Pseudomonas syringae*		
Total area of the damaged tissue (necrotic lesions) after 6 days (cm^2)		Percent reduction in disease incidence	Total area of the damaged tissue (necrotic lesions) after 6 days (cm^2)		Percent reduction in disease incidence
Untreated	*Pre-treated		Untreated	*Pre-treated	
100.3±1.55	11.7±1.00	88.33±1.55	115.7±1.04	9.6±0.90	91.7±0.92

*data of 100 spots in 10 fruits. No lesion was found in control (untreated-uninoculated) tomatoes

In vivo application of botanicals and assessment of disease incidence in the pineapple fruits following challenge inoculation by the pathogens have also been performed like the other two fruits. Results (Table: 4.27) revealed that *A. alternata* (isolate AaP) caused disease in the untreated-inoculated fruits. Total area of the fruits affected was 48.1 cm^2 but when bioactive fraction of *Xanthium strumarium* leaf extract

(concentration of 100µg/ml) was applied prior to inoculation by the fungal pathogen, 85.23% disease was reduced. Under similar conditions disease incidence caused by *Erwinia* sp.was reduced up to 90.42%.

Table 4.26: Pathogenicity of *A.alternata* (isolate AaP) and *Erwinia* sp. on pineapple collected from different places of North Bengal

Pineapple collected from	Inoculated by *A. alternata* (isolate AaP)		Inoculated by *Erwinia* sp.	
	Percent lesion formed after 6 days	Total area of the damaged tissue (necrotic lesion) after 6 days * (cm^2)	Percent lesion formed after 6 days	Total area of the damaged tissue (necrotic lesion) after 6 days * (cm^2)
Fatapukur	30±0.58	39±0.64	39±0.95	50±0.75
Chopra	23±0.60	29±0.75	37±1.84	48±2.14
Bidhannagar	37±0.62	48±0.70	51±1.25	86±0.95
Ghoshpukur	34±0.62	44±0.92	43±1.15	73±0.85
CD5%	0.24	1.10	1.21	0.50

* Data of 100 spots in 5 fruits. No lesion was found in control pineapples.

Table 4.27: Diseases incidence (total area of necrotic lesions) following application of *Xanthium strumarium* leaf extracts in susceptible pineapples collected from Bidhannagar area.

Inoculated by *A. alternata* (AaP)			Inoculated by *Erwinia* sp.		
Total area of the damaged tissue (necrotic lesions) after 6 days		Percent reduction in disease incidence	Total area of the damaged tissue (necrotic lesions) after 6 days		Percent reduction in disease incidence
Untreated	*Pre-treated		Untreated	*Pre-treated	
48.1cm^2±3.28	7.1±0.91	85.23±0.91	86.7±1.21	8.3±0.32	90.42±1.57

*data of 100 spots in 5 fruits. No lesion was found in control (untreated-uninoculated) pineapples.

4.11: Isolation of bacterial and fungal isolates antagonistic to microbial pathogen(s)

With an objective to find out some antagonistic soil bacteria and fungi several soil bacteria and fungi were isolated from the rhizosphere of tea plants and also from rhizosphere of forest trees of North Bengal. Altogether, 31 bacterial and 19 fungal isolates were found in pure form. Isolation and purification procedures have been described in details in materials and methods (section: 3.2.3). The isolated bacterial cultures were coded as SB1-SB31 (SB stands for soil bacteria) and fungal cultures were coded as SF1-SF19 (SF stands for soil fungi). These isolates were screened for the presence of biocontrol activity against three fungal pathogens [viz. *Fusarium moniliforme, Alternaria alternata* (isolate AaT) *and Alternaria alternata* (isolate AaP)] and three bacterial pathogens [viz. *Xanthomonas* sp. *Pseudomonas syrinage* and *Erwinia* sp.]. The details of the results have been presented in the table (4.28), table (4.29) and table (4.30), [plate 19 to 25].

Table 4.28: *In vitro* antagonistic activity of bacterial isolates against selected fungal pathogens (following dual culture test).

Bacterial antagonist	Fungal pathogens					
	Fusarium moniliforme		*Alternaria alternata* (AaT)		*Alternaria alternata* (AaP)	
	Mycelial diameter (mm)	% inhibition of growth *	Mycelial diameter (mm)	% inhibition of growth	Mycelial diameter(mm)	% inhibition of growth
SB1	27	70.00	35	61.11	15	76.66
SB2	25	72.22	33	63.33	11	74.44
SB3	60	33.33	48	46.66	42	53.33
SB4	41	54.44	39	56.66	51	43.33
SB5	63	30.00	61	32.22	50	44.44
SB6	39	56.66	35	61.11	40	55.55
SB7	45	50.00	32	64.44	36	60.00
SB8	59	34.44	45	50.00	37	58.88
SB9	48	46.66	49	45.55	40	55.55
SB10	41	54.44	49	45.55	38	57.77
SB11	39	56.66	55	38.88	47	47.77
SB12	43	52.22	43	52.22	42	53.33
SB13	52	42.22	42	53.33	40	55.55
SB14	62	31.11	49	45.55	32	64.44
SB15	61	32.22	53	41.11	59	34.44
SB16	49	45.55	63	30.00	48	46.66
SB16	40	55.55	49	45.55	62	31.11
SB17	38	57.77	41	54.44	34	62.22
SB18	47	47.77	65	27.77	60	33.33
SB19	51	43.33	43	52.22	49	45.55
SB20	69	23.33	33	63.33	30	66.66
SB21	59	34.44	49	45.55	40	55.55
SB23	72	20.00	28	68.88	30	66.66
SB24	68	24.44	43	52.22	43	52.22
SB25	56	37.77	42	53.33	33	63.33
SB26	51	43.33	62	31.11	59	34.44
SB27	39	56.66	68	24.44	50	40.00
SB28	54	40.00	51	43.33	57	36.66
SB29	40	55.55	50	40.00	43	52.22
SB30	34	62.22	34	62.22	40	55.55
SB31	33	63.33	37	58.88	32	64.44

SB=Soil bacteria; *Percent inhibition growth in relation to control = [(Diameter of growth of fungi in control plate - Diameter of growth of fungi in dual culture plate)/ Diameter of growth of fungi in control plate] X 10

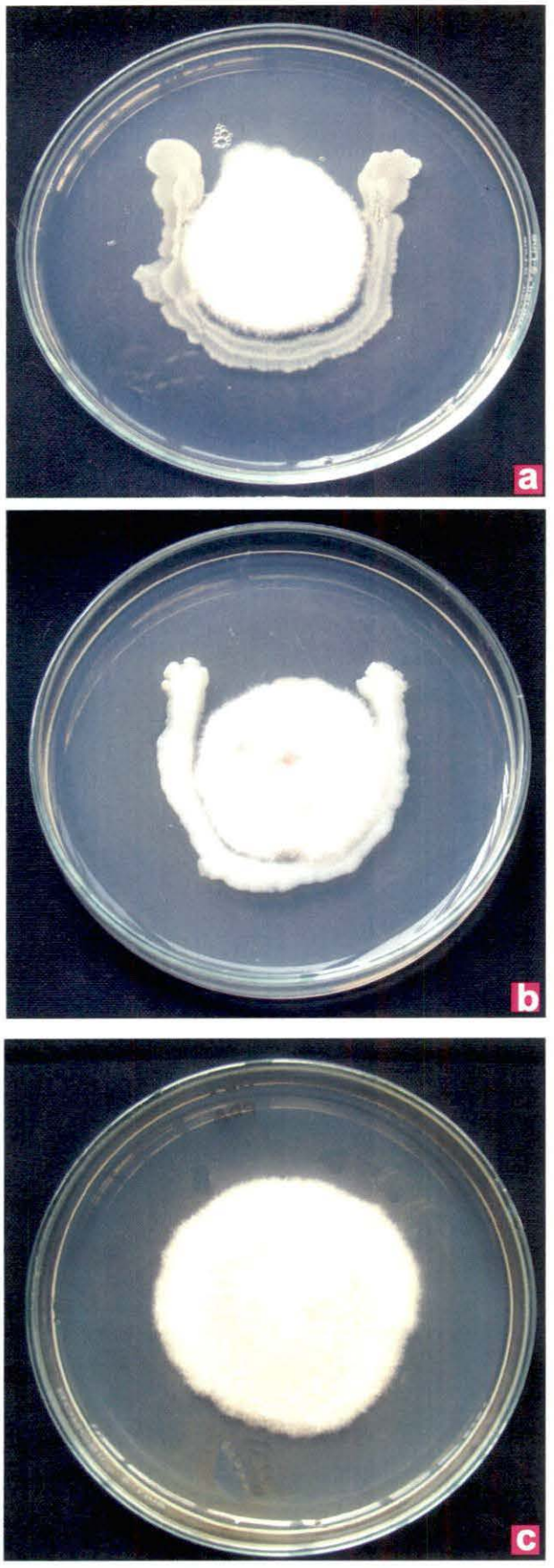


Plate 19: Bio control by antagonistic microrganism **fig. a:** Dual culture of *Lysinibacillus sphaericus* and *Fusarium moniliforme*
fig. b: Dual culture of *Pseudomonas* sp. and *F. moniliforme*
fig. c: *F. moniliforme* in PDA (Control)

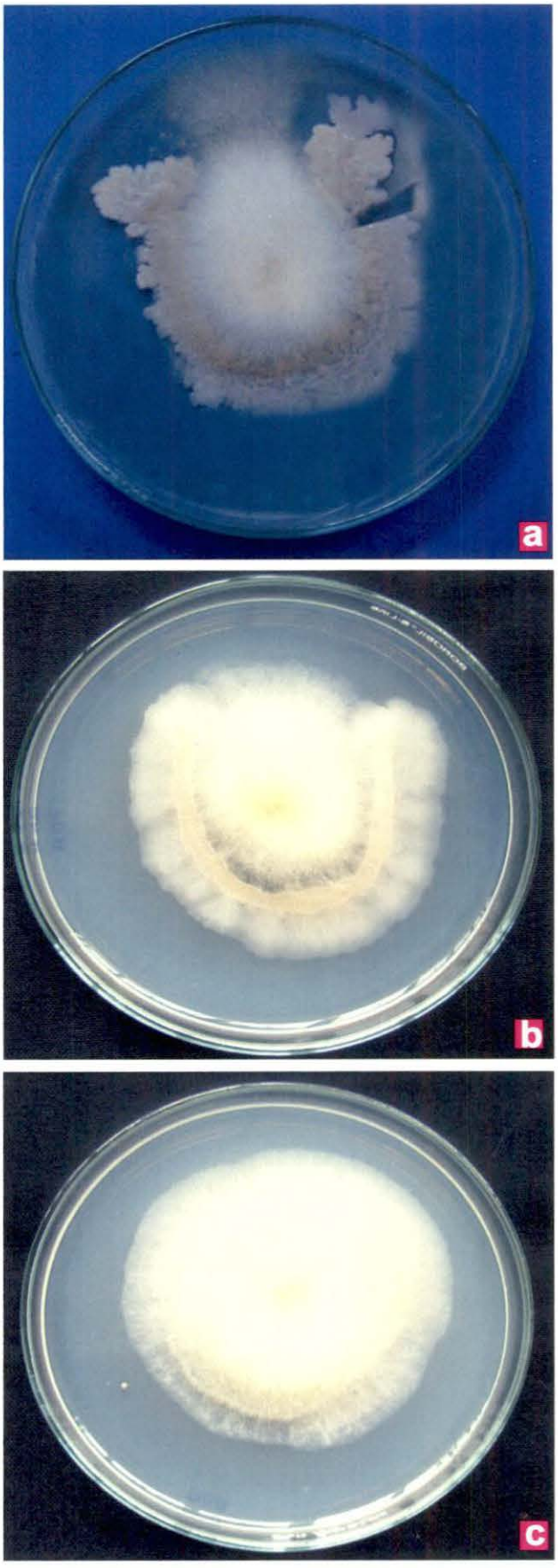


Plate 20: Bio control by antagonistic microrganism **fig. a:** Dual culture of *Lysinibacillus sphaericus* and *Alternaria alternata* (AaT) **fig. b:** Dual culture of *Pseudomonas* sp. and *A. alternata* (AaT) **fig. c:** *A. alternata* (AaT) in PDA (Control)

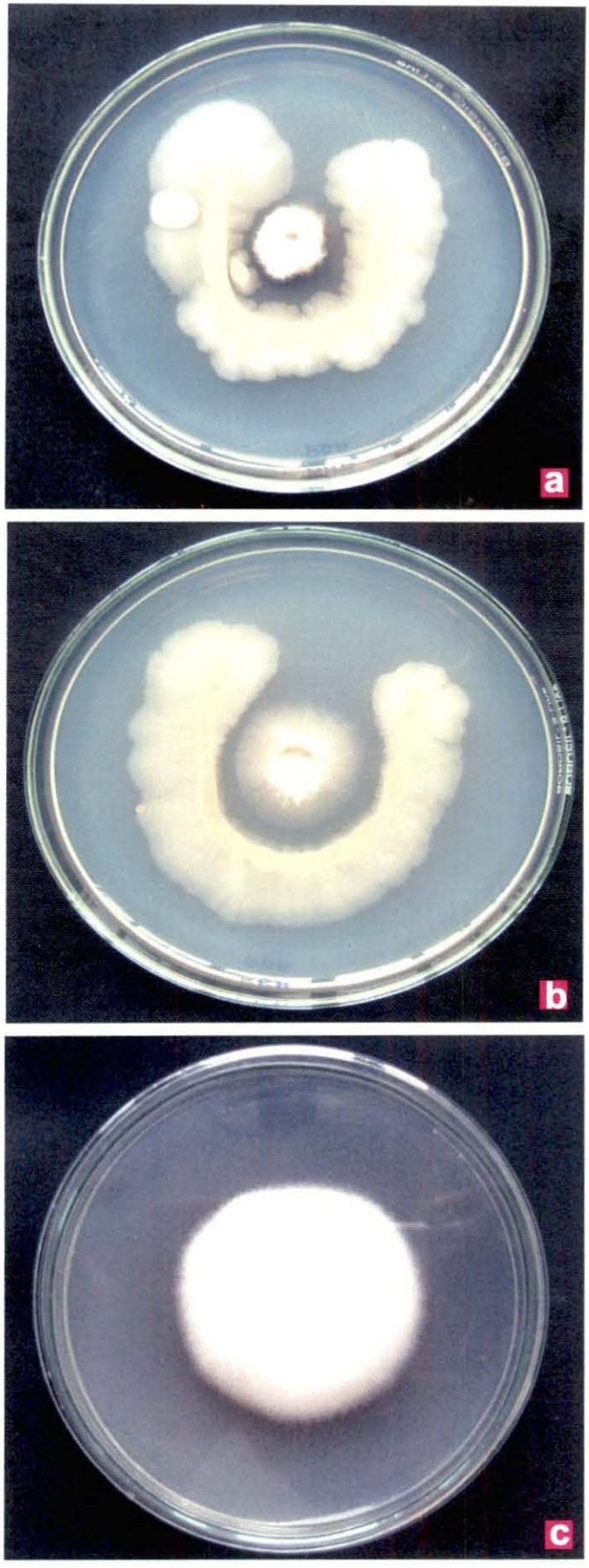


Plate 21: Bio-control by antagonistic microrganism **fig. a:** Dual culture of *Lysinibacillus sphaericus* and *Alternaria alternata* (AaP) **fig. b:** Dual culture of *Pseudomonas* sp. and *A. alternata* (AaP) **fig. c:** *A. alternata* (AaP) in PDA (Control)

Table 4.29: *In vitro* antagonistic activity of isolated (from soil) bacterial isolates against selected bacterial pathogens.

Bacterial Antagonist	Bacterial pathogens					
	Xanthomonas sp.		*Pseudomonas syringae*		*Erwinia* sp.	
	Growth at cross point of two bacterial streaks (mm)	% inhibition of growth in relation to control	Growth at cross point of two bacterial streaks (mm)	% inhibition of growth in relation to control	Growth at cross point of two bacterial streaks (mm)	% inhibition of growth in relation to control
SB1	8	77.14	9	76.92	8	77.77
SB2	4	88.57	7	84.61	5	86.11
SB3	24	31.42	19	51.28	16	55.55
SB4	17	51.42	21	46.15	23	36.11
SB5	12	65.71	17	56.41	15	58.33
SB6	16	54.28	28	28.20	21	41.66
SB6	19	45.71	33	15.38	24	33.33
SB7	12	65.71	12	69.23	15	58.33
SB8	14	60.00	28	28.20	25	30.55
SB9	10	71.42	20	48.71	26	27.77
SB10	15	57.14	30	23.07	21	41.66
SB11	27	22.85	31	20.51	20	44.44
SB12	29	17.14	26	33.33	27	25.00
SB13	13	62.85	10	74.35	13	63.88
SB14	13	62.85	17	56.41	10	72.22
SB15	22	37.14	28	28.20	24	33.33
SB16	31	11.42	29	25.64	25	30.55
SB16	32	08.57	34	12.82	24	33.33
SB17	27	22.85	29	25.64	23	36.11
SB18	21	04.00	18	53.46	17	52.77
SB19	11	68.57	15	61.53	15	58.33
SB20	15	57.14	23	41.02	20	44.44
SB21	17	51.42	28	28.20	22	38.88
SB22	12	65.71	16	58.97	19	47.22
SB23	19	45.71	29	25.64	23	36.11
SB24	16	54.28	21	46.15	24	33.33
SB25	14	60.00	20	48.71	16	55.55
SB26	11	68.57	16	58.97	12	66.66
SB27	15	57.14	21	46.15	22	38.88
SB28	24	31.42	29	25.64	20	44.44
SB29	10	71.42	15	61.53	12	66.66
SB30	31	11.42	30	23.07	23	36.11
SB31	24	31.42	29	25.64	24	61.11

SB=Soil bacteria

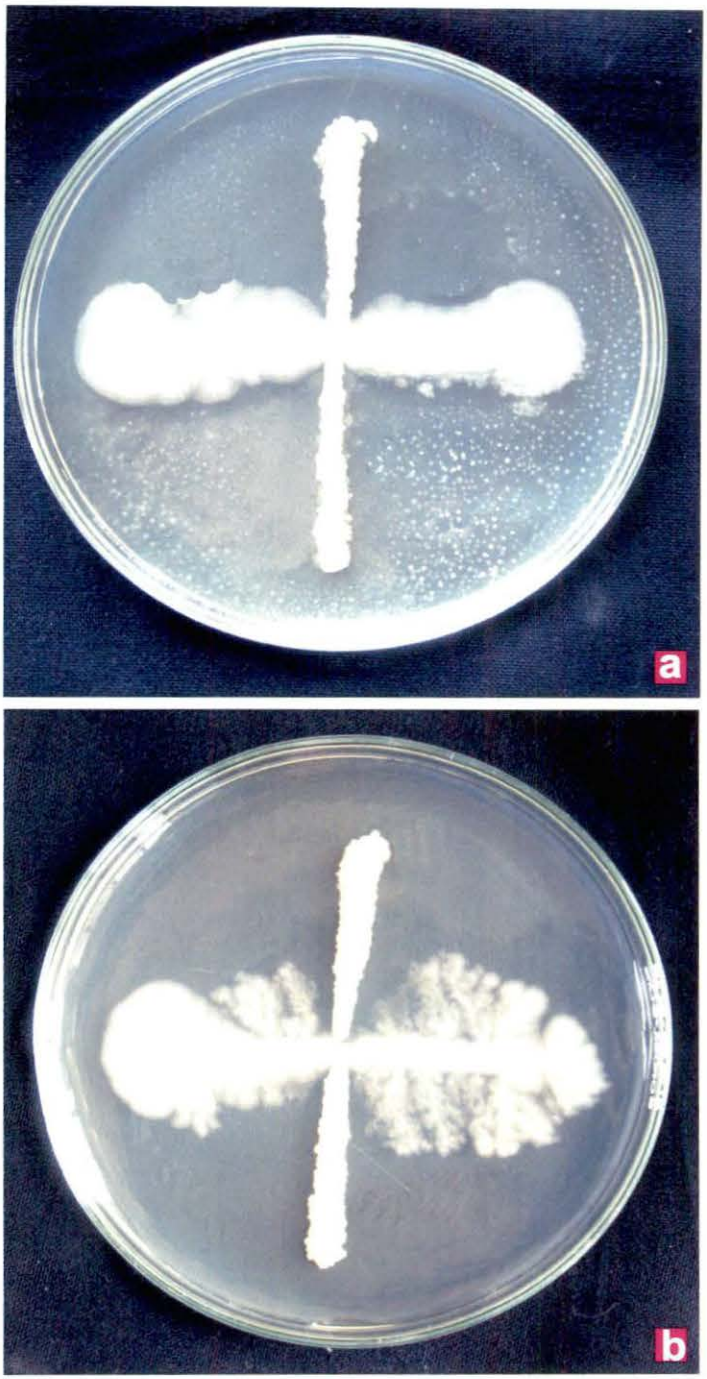


Plate 22: Dual culture of antagonistic bacteria and pathogen (*Xanthomonas* sp.)
fig.a: Dual culture of bacterial pathogen *Xanthomonas* sp. (streaked horizontally) and *Lysinibacillus sphaericus* (streaked vertically)
fig.b: Dual culture of bacterial pathogen *Xanthomonas* sp. (streaked horizontally) and *Pseudomonas* sp. (streaked vertically)

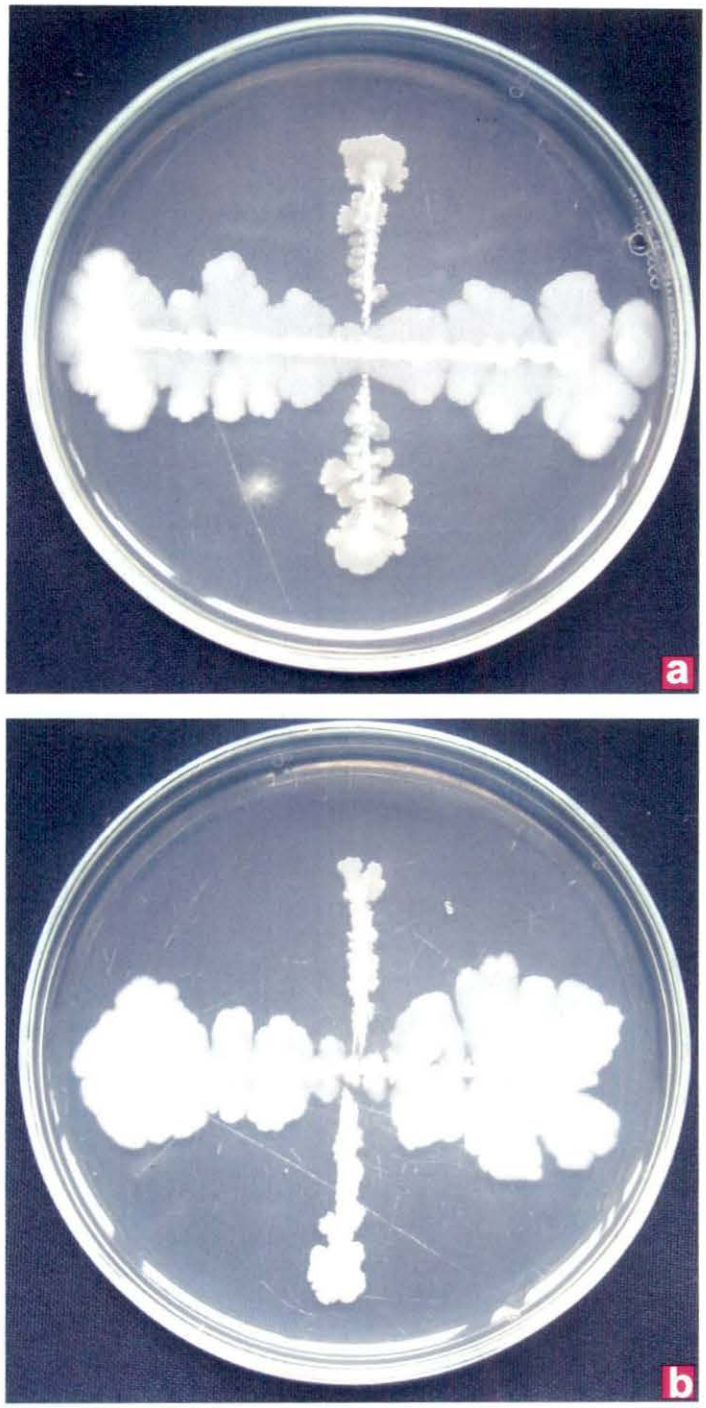


Plate 23: Dual culture of antagonistic bacteria and pathogen (*Pseudomonas* sp.)

fig.a: Dual culture of bacterial pathogen *Pseudomonas* sp.(streaked horizontally) and *Lysinibacillus sphaericus* (streaked vertically)

fig. b: Dual culture of bacterial pathogen *Pseudomonas syringae* (streaked horizontally) and *Pseudomonas* sp. (streaked vertically).

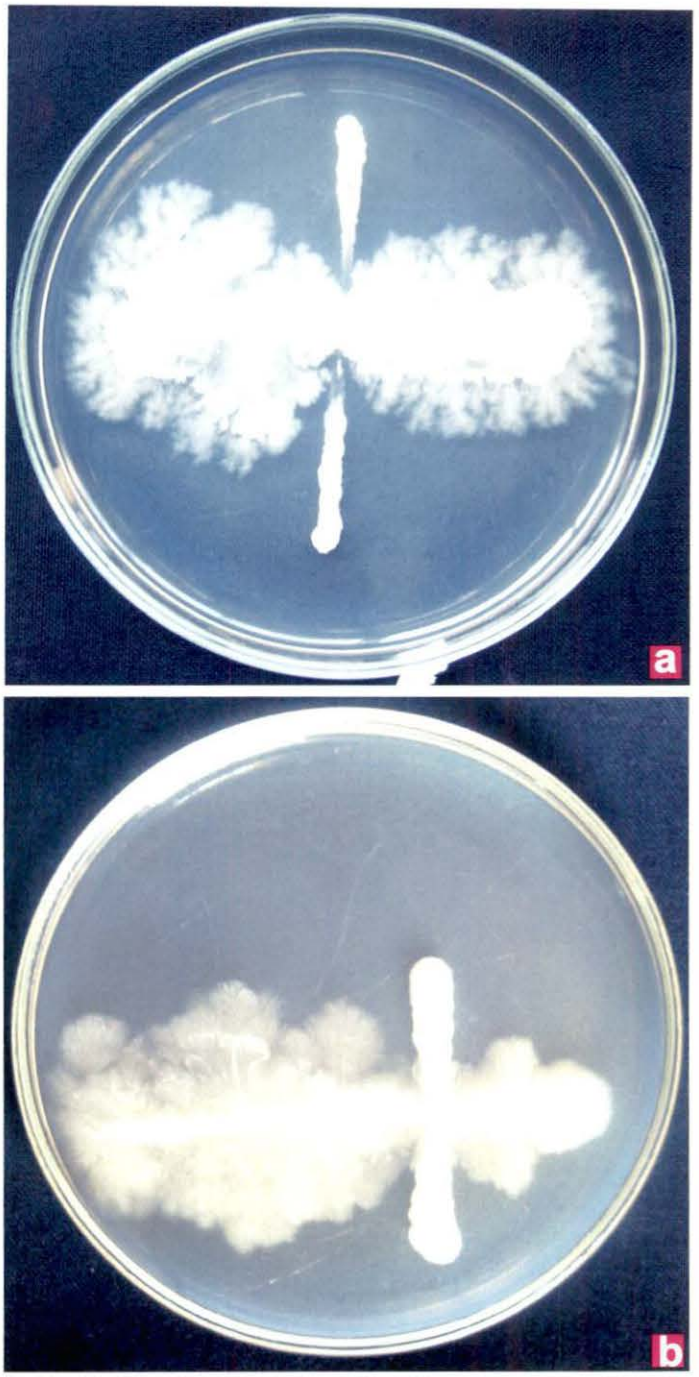


Plate 24: Dual culture of antagonistic bacteria and pathogen (*Eriwina* sp.)
fig.a: Dual culture of bacterial pathogen *Eriwina* sp. (streaked horizontally) and *Lysinibacillus sphaericus* (streaked vertically)
fig.b: Dual culture of bacterial pathogen *Eriwina* sp. (streaked horizontally) and *Pseudomonas* sp. (streaked vertically)

Table 4.30: *In vitro* antagonistic activity of fungal isolates against selected fungal pathogens.

FUNGAL ANTAGONISTS	*Fusarium moniliforme*		*Alternaria alternata* (isolate AaT)		*Alternaria alternata* (isolate AaP)	
	Mycelial diameter in mm *	% inhibition of growth in relation to control	Mycelial diameter in mm *	%inhibition of growth in relation to control	Mycelial diameter in mm*	% inhibition of growth in relation to control
SF1	13	85.55	34	62.22	9	90.00
SF2	23	74.44	46	48.88	29	67.77
SF3	29	67.66	48	46.66	32	64.44
SF4	56	37.77	54	40.00	48	46.66
SF5	45	50.00	56	37.77	38	57.77
SF6	59	34.44	19	78.88	21	76.66
SF7	17	81.11	25	72.22	14	84.44
SF8	48	46.66	67	25.55	66	26.66
SF9	63	30.00	45	50.00	39	56.66
SF10	23	74.44	28	68.88	36	60.00
SF11	30	66.66	30	66.66	47	47.77
SF12	58	35.55	54	40.00	49	45.55
SF13	41	54.44	60	33.33	48	46.66
SF14	32	64.44	62	31.11	54	40.00
SF15	51	43.33	59	43.33	62	31.11
SF16	44	51.11	61	32.22	30	66.66
SF17	22	75.55	42	53.33	33	63.33
SF18	51	43.33	39	56.66	61	32.22
SF19	68	57.77	43	52.22	41	54.44

Data given are after 4 days of inoculation.

One potential antagonistic fungal genera (SF7) was identified in the laboratory and the identifications were confirmed by sending them to Indian Type Culture Collection, IARI, New Delhi. SF7 was identified as *Aspergillus flavus. Trichoderma harzianum* (previously identified from IARI, NEW DELHI) was collected from molecular plant pathology and fungal biotechnology laboratory and was used in the present study. The two soil bacteria (SB1 and SB2) were also identified. SB1 and SB2 have been identified (by biochemical tests) as *Bacillus* sp and *Pseudomonas* sp. The 16S rDNA studies of SB1 (*Bacillus* sp) have confirmed the bacteria as *Lysinibacillus sphaericus*. The details of the molecular studies have been presented in the section: 3.6.5.

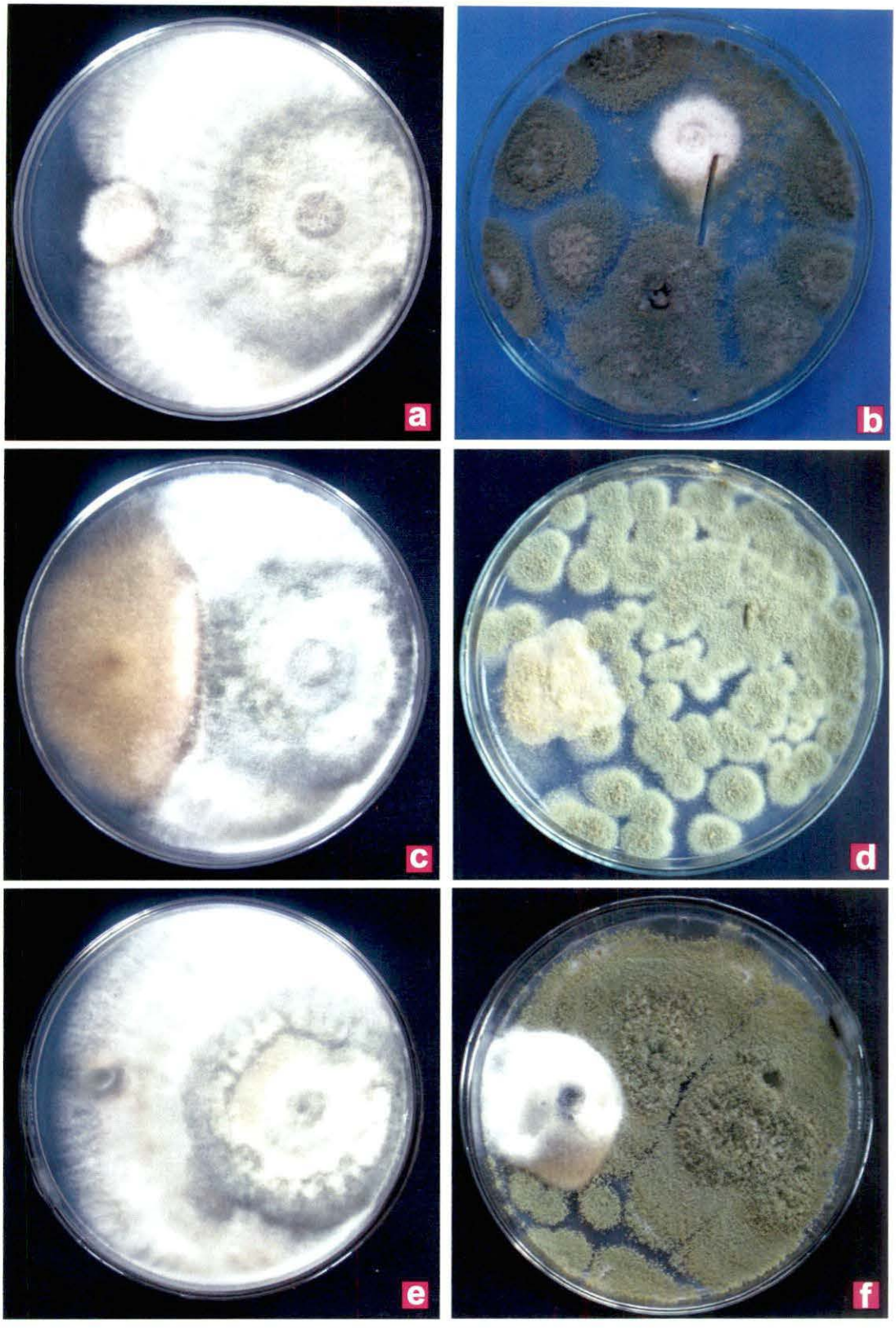


Plate 25: Bio-control by antagonistic microrganism **fig.a:** Dual culture of *Fusarium moniliforme* and *Tricoderma harzianum* **fig.b:** Dual culture of *F.moniliforme* and *Aspergillus flavus* **fig.c:** Dual culture of *Alternaria alternata* (AaT) and *Tricoderma harzianum* **fig.d:** Dual culture of *A.alternata* (AaT) and *A. flavus* **fig.e:**Dual culture of *A.alternata* (AaP) and *T.harzianum* **fig.f:** Dual culture of *A.alternata* (AaP) and *A. flavus.*

In the present study *Trichoderma harzianum* and *Aspergillus flavus* and two bacterial strains [SB1 (*Lysinibacillus sphaericus*) and SB2 (*Pseudomonas* sp.)] were used for their efficacy against selected fungal pathogens [*Fusarium moniliforme*, *Alernaria alternata* (isolate AaT) and *Alernaria alternata* (isolate AaP)] and bacterial pathogens (*Xanthomonas* sp., *Psedomonas syringae* and *Erwinia* sp.). Fungal antagonist (*Trichoderma harzianum* and *Aspergillus flavus*) and bacterial isolates (*Lysinibacillus sphaericus* and *Psedomonas* sp.) were isolated from soil and selected through dual culture for the present study in controlling post harvest pathogens. *Trichoderma harzianum* and *Aspergillus flavus* inhibited *Fusarium moniliforme*, *Alernaria alternata* (AaT) and *Alernaria alternata* (AaP) in dual cultures. Results of dual cultures involving SB1 (*Lysinibacillus sphaericus)* or SB2 (*Psedomonas* sp.) as one of the cultures against the pathogens (separately) clearly indicated that SB2 (*Psedomonas* sp.) is the best biocontrol agent among the two bacterial antagonists. SB2 inhibited (88.57%) growth of *Xanthomonas* sp. (plate: 22, fig. b). SB1 inhibited(77.14%) growth of *Xanthomonas* sp. (Plate:22, fig. a). SB1 also inhibited growth (61.11%) of *Alternaria alternata* (AaP) [plate: 20, fig. a]. *Alternaria alternata*(AaT) was least inhibited by SF1 (plate: 25, fig. c). *Trichoderma harzianum* showed highest inhibition (90.00%) over *Alternaria alternata*(AaP), [plate: 25, fig. e]. Results of the dual culture involving the four antagonists clearly indicated that SF1 is the best biocontrol agent.

4.12: Molecular characterization of antagonist bacteria (16S rDNA gene sequencing):

A PCR product of approximately 1.5 kb was generated from the bacteria SB1. Sequencing of the 16S rDNA gene followed by BLAST searches revealed that SB1 (99%) was similar to *Lysinibacillus sphaericus* strains. Sequence similarities between the isolate and other *Lysinibacillus* and *Bacillus* strains varied from 98% to 99%. In blast search analysis based on these sequences, the present isolate was identified as *Lysinibacillus sphaericus.* (Plate: 26, fig. b)

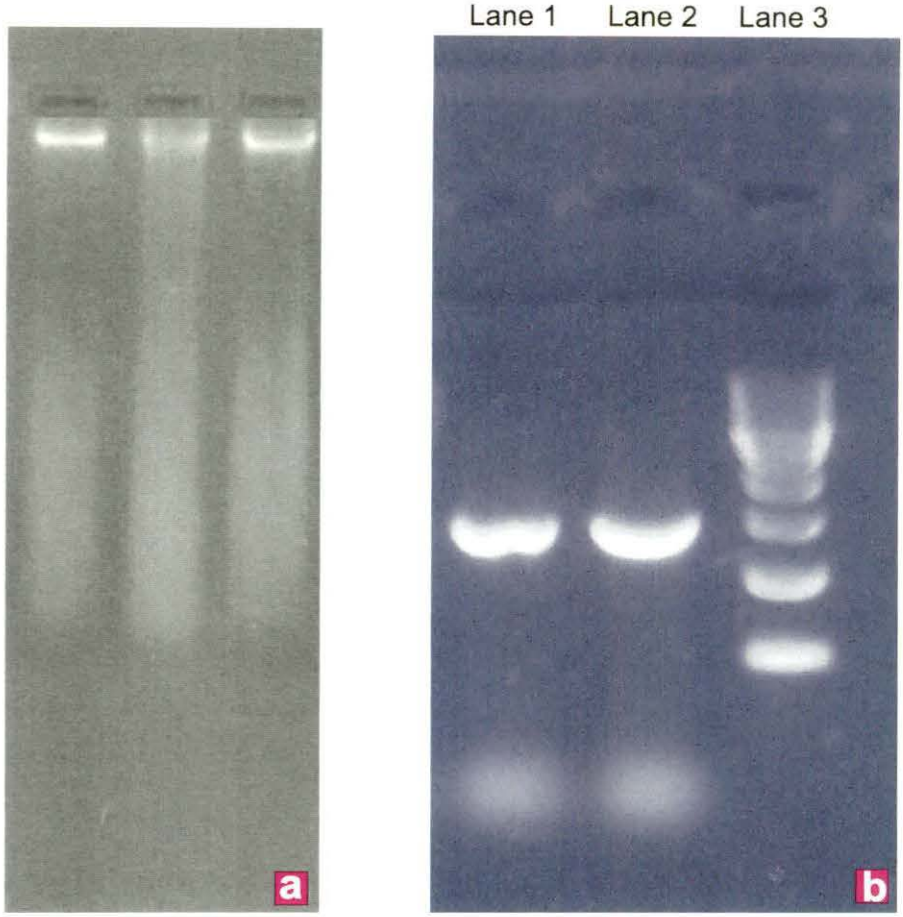


Plate 26 : fig.a: Detection of genomic DNA of bacteria isolates in agarose gel.
fig.b: Agarose gel electrophoresis of 16S rDNA amplified products. Lanes:*Lysinibacillus sphaericus,*1; *Pseudomonas* sp.,2; 500 bp DNA ladder, 3.

4.13: Effect of crude culture filtrates of fungal antagonists on the growth of *Fusarium moniliforme*, *Alternaria alternata* (AaT) and *Alternaria alternata* (AaP)

Three fungal pathogens were allowed to grow separately in PDA mixed with culture filtrates (9:1) of *Trichoderma harzianum* and *Aspergillus flavus* as described under materials and methods (Section: 3.5.4.). Radial growth of the fungal pathogens was measured after 3 days of inoculation. Percent inhibitions in relation to control were calculated and the results were tabulated in table (4.31). From results it was evident that culture filtrate of both antagonists (viz. *T. harzianum* and *A. flavus*) completely inhibited the growth of the pathogens.

Table 4.31: *In vitro* effect of crude culture filtrates of fungal antagonists on the growth of *F. moniliforme*, *A. alternata* (AaT) and *A. alternata* (AaP)

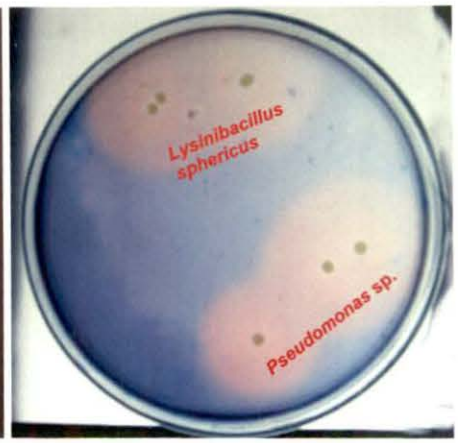
Culture filtrate of fungal antagonists	*Fusarium moniliforme*		*Alternaria alternata*(AaT)		*Alternaria alternata*(AaP)	
	Radial growth (diameter) mm	% inhibition of growth	Radial growth (diameter) mm	% inhibition of growth	Radial growth (diameter) mm	% inhibition of growth
Trichoderma harzianum	00	100	00	100	00	100
Aspergillus flavus	00	100	00	100	00	100

* Mean of three replications. Data are after 4 days of inoculation. *** PDA: Culture filtrate = 9:1. Culture filtrates were collected from 15 days old culture. **Control diameter = 9.0 cm after 10 days of inoculation.

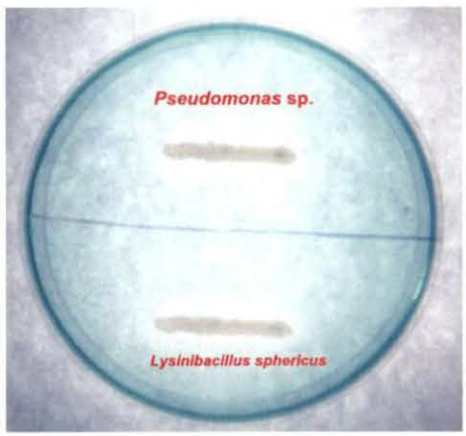
4.14: Characterization of antimicrobial metabolites produced by the bacterial and fungal antagonists:

Trichoderma harzianum, Aspergillus flavus, Lysinibacillus sphaericus and *Pseudomonas* sp. were tested for Pectinase, phosphatase and DNase activity. None of the tested organisms showed pectinase and phosphatase (Table: 32 & 33). All the four organisms were also subjected to cellulase production test, chitinase production test and siderophore production test. All the four organism exhibited cellulase activity (plate: 27). *Aspergillus flavus, Lysinibacillus sphaericus* and *Pseudomonas* sp. produced siderophore

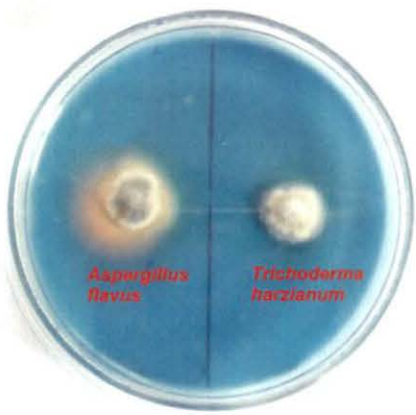
Lipase Production test



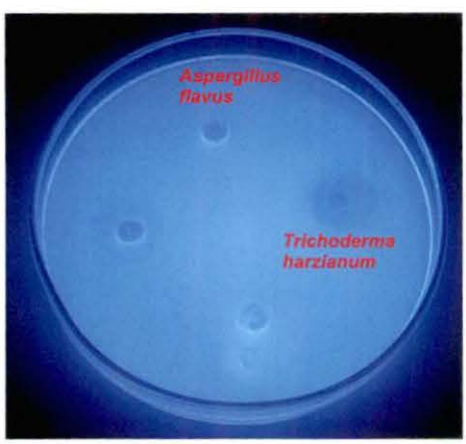
Siderophore production by bacterial antagonists



DNase Production test



Siderophore production by fungal antagonists



Chitinase Production test

Plate 27: Antimicrobial metabolites produced by bacterial and fungal antagonists.

4.15. Scanning Electron Microscopic studies:

Scanning electron microscopic studies of the interacting zones of the antagonistic microorganisms and pathogens were performed. The results of the study were recorded in photographs presented in Plates 28, 29 & 30. From the plate: 28 (fig. d and fig. e) it is clear that spore(s) for the antagonistic fungus (*A. flavus*) attached with the hyphae of the pathogen *F. moniliform e*. In the same plate in fig f, g & h we get interaction of *Pseudomonas* sp. with *F. moniliform e.* Both the antagonistic organisms degraded the hyphae of the pathogen and checked the growth of the pathogen (*F. moniliform e*).

In plate: 29, fig. b, c & fig. d show that spore(s) of *A. flavus* is attached with the hyphae of the pathogen *Alternaria alternata* (isolate AaT) and damaged the hyphal walls. In the same plate (fig. e and fig. f) we get *Pseudomonas* sp is attached with the hyphae of *Alternaria alternata* (isolate AaT). Prominent damaged hyphae show cluster of bacteria.

In plate 30: fig.c shows spores of *Aspergillus flavus* attached with *Alternaria alternata* (isolate AaP). Similarly fig d shows attachment of *Pseudomonas* sp. with the hyphae of the pathogen *Alternaria alternata* (isolate AaP).

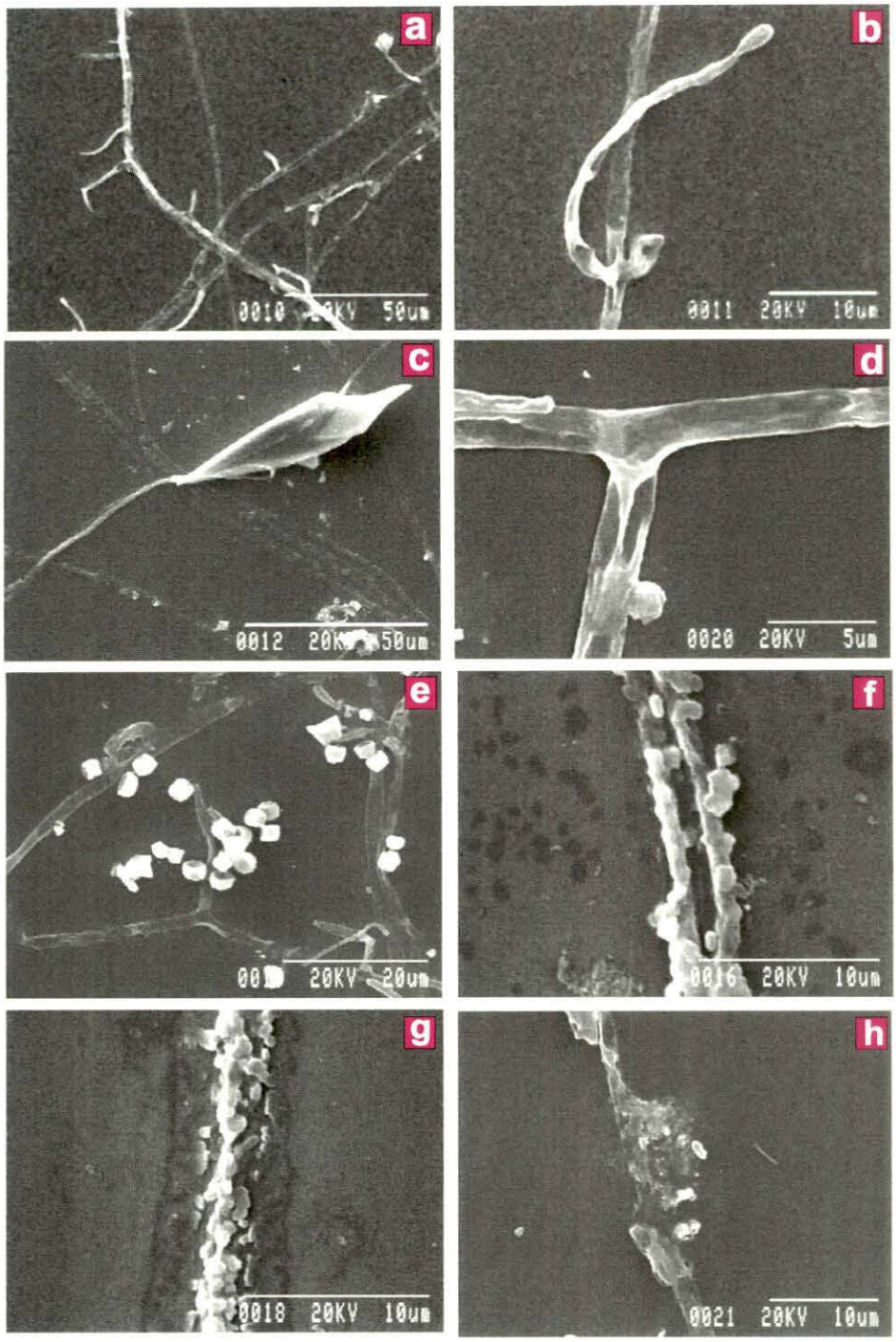


Plate 28: Scanning electron micrographs. **fig.a:** Hyphae of *Fusarium moniliforme*; **fig.b:** Hyphal branching of *F. moniliforme;* **fig.c:** Macroconidia of *F. moniliforme* **fig.d:** One spore of *Aspergillus flavus* attached with hyphae of *F. moniliformr;* **fig.e:** Spores of *A. flavus* interacting with hyphae of *F. moniliforme;* **fig. f,g&h:** Antagonistic bacteria *Pseudomonas* sp. interacting with *F. moniliforme.*

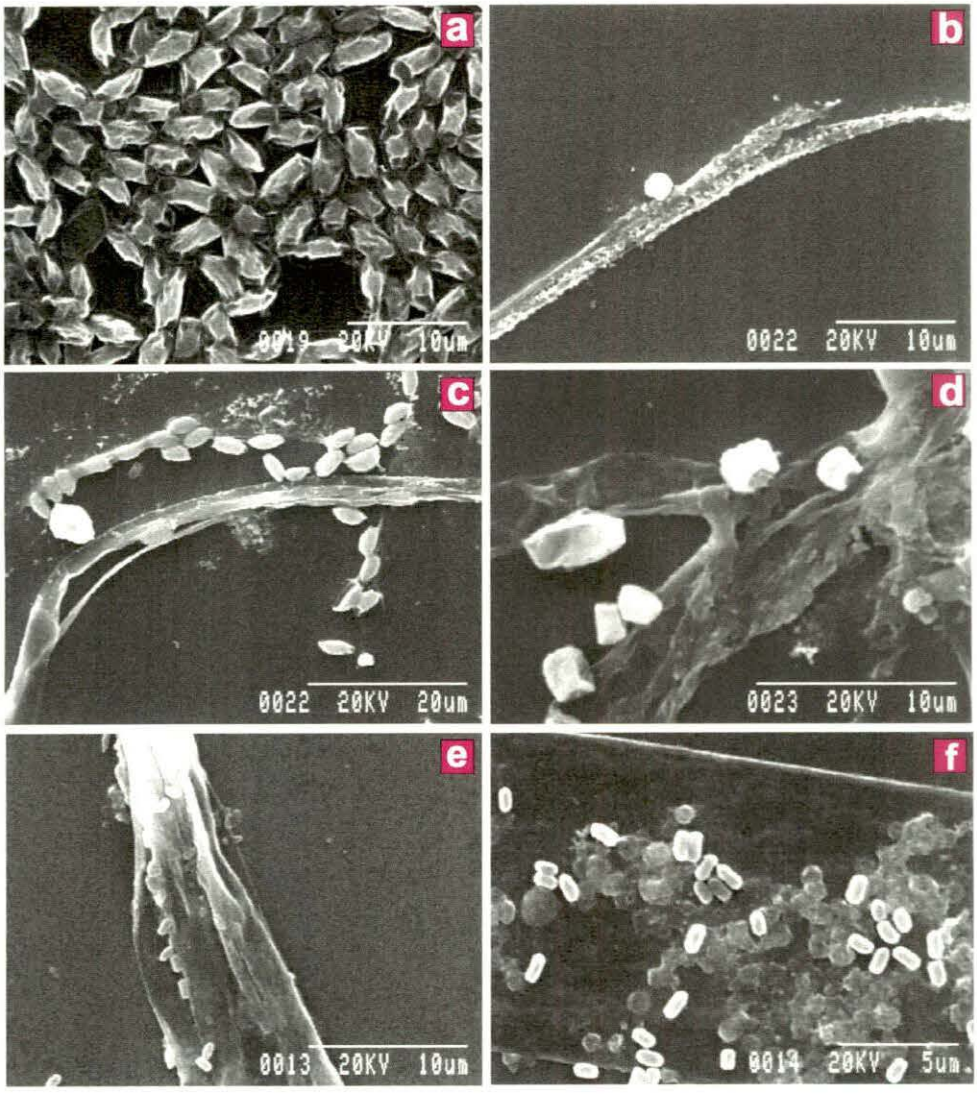


Plate 29: Scanning electron micrographs. **fig.a:** Spores of *Alternaria alternata* (AaT);
fig.b,c&d: Spores of *Aspergillus flavus* attached with hyphae of *Alternaria alternata* (AaT);
fig.e&f: Antagonistic bacteria *Pseudomonas* sp. attached with the hyphae of *A. alternata* (AaT).

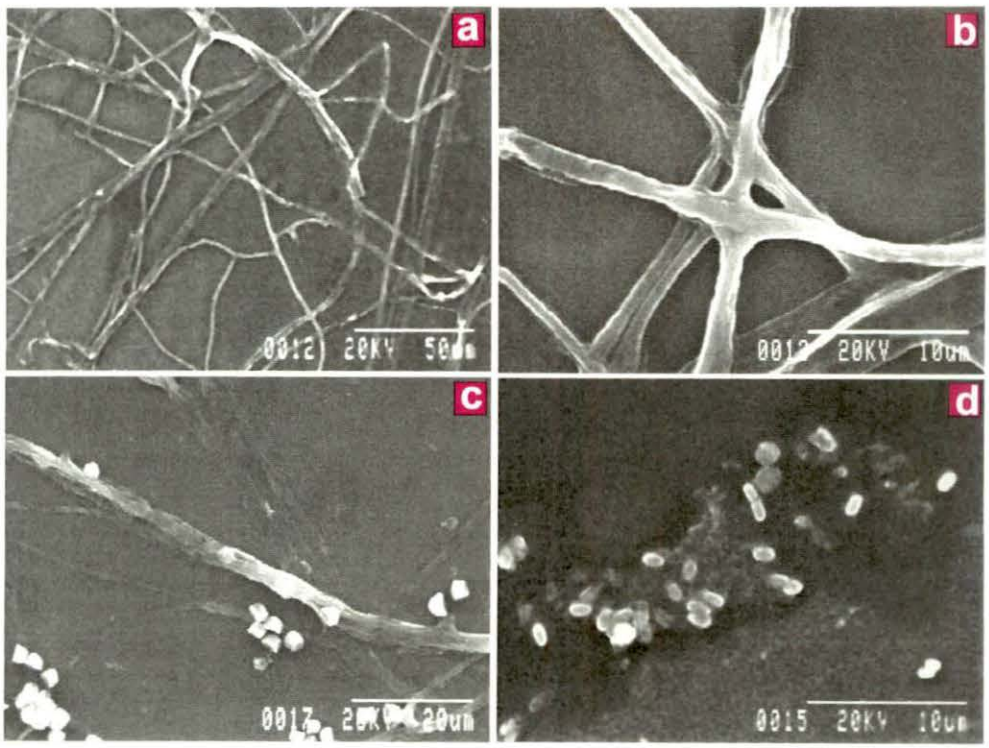


Plate 30: Scanning electron micrographs. **fig.a&b:** Hyphae of *Alternaria alternata* (AaP);
fig.c: Spores of *Aspergillus flavus* attached with hyphae of *Alternaria alternata* (AaP);
fig.d: Antagonistic bacteria *Pseudomonas* sp. attached with the hyphae of *A. alternata* (AaP).