5. Discussion

India loses about 35-40% of the produce due to improper post harvest management. A loss estimated at Rs 40,000 crores per year! India wastes fruits and vegetables every year equivalent to the annual consumption of the United Kingdom (Anonymous, 2008).

Since, fruits and vegetables are highly perishable, efficient post harvest management has become an absolute necessity. In the present study three different fruits and vegetables have been taken into consideration for post harvest disease control. The three fruits and vegetables are orange (one of the major citrus fruit), tomato (although it is a fruit but is considered as vegetable) and pineapple. In a developing country like India, post-harvest losses of citrus fruits are in the range of 25-30% as against 5-10% in developed citrus growing countries like Brazil, USA, Australia, Spain, Italy and Israel. This is mainly due to the unscientific practices of picking, handling, packaging, transport and storage (Sonkar *et al.*, 2008).

Orange (*Citrus reticulata*), tomato (*Lycopersicon esculentum*) and pineapple (*Ananas comosus*) are major economically important fruits and vegetables of North Bengal (Sub-Himalayan West Bengal). Orange, tomato and pineapple also grow well in different parts of North Bengal. With the increase in population and market demand, an increase in production of the fruits has also been noticed in recent years. Post harvest disease problems associated with these juicy fruits and vegetables have also been increased. Storage life of fruits and vegetables largely depend upon factors like cultivar, harvest timing, source, growing season, climate and nutritional conditions. A small loss can be very expensive because of the accumulated cost of growing, harvesting and storing of these high value commodities.

Proper post harvest disease management is an important aspect of successful marketing of any crop. Like many other fruits and vegetables, orange, tomato and pineapple are also subject to post harvest diseases caused by many fungi and bacteria. The application of broad spectra of fungicides was the common practice in almost for all the plants to control microbial diseases. The fungicides are extremely hazardous to our health and environment. Therefore it's essential to adopt eco-friendly methods and to

control diseases caused by microbes and botanicals. With these observations the present work was under taken and it is likely that the results will broaden the scientific base upon which total control of post harvest diseases of orange, tomato and pineapple may be established through an integrated post harvest disease management approach. At the onset of the study, several fungi and bacteria were isolated from post harvest diseased fruits of orange, tomato and pineapple.

Fusarium moniliforme and *Xanthomonas* sp. were isolated from orange collected from Garubathan market situated in Jalpaiguri, North Bengal. The post harvest diseases caused by the above mentioned two pathogens were subjected to control in the present study. Eckert and Brown (1986) have reported that the diseases were present in all cultivars and in most citrus growing areas. It was also reported that long-term cold storage of the fruits was troublesome due to disease problems. *Xanthomonas* sp. found throughout all citrus growing regions. Citrus plant is attacked by a number of diseases like citrus canker, gummosis, citrus decline and greening etc. But citrus canker caused by the bacterium *Xanthomonas* sp., is probably the worst enemy to the citrus plantations as stated by Awan *et al.,* 1992. Asiatic citrus canker induced by *X. axonopodis* pv. *citri* has re-emerged as potential threat to citrus plantation throughout the world (Gottwald *et al.*, 2001). The incipient infection of pre-harvest pathogens subsequently also manifest in the form of post-harvest diseases besides the attack of other post-harvest wound pathogens viz. *Penicillium digitatum, P. italicum, Geotrichum candidum, Fusarium moniliforme and Xanthomonas citri* etc (Naqvi, 2004).

Alternaria alternata (isolate AaT) and *Pseudomonas* sp. were isolated from tomato collected from Haldibari (Coochbehar), a place situated in North Bengal and the place is renowned for cultivation of tomato. The two pathogens were used throughout the present study as test pathogen of post harvest diseases of tomato. Postharvest decay is the major limiting factor for extension of shelf life in tomato fruits. *Alternaria alternata* and *Botrytis cinerea* causing black and grey moulds are the two main fungi responsible for storage decay (Barnett and Hunter, 1998; Tohamy *et al.*, 2004). Tomato commercialization is limited by rotting caused by *Alternaria alternata* or by *Botrytis cinerea* (Jones *et al.*, 1993). Postharvest losses due to Bacterial Soft Rot (*Erwinia*, *Pseudomonas* and others), *Geotrichum* Sour Rot, *Rhizopus* Rot and *Botrytis* Gray Mold

were greater (Cantwell and Nie, 1996; Mitcham and Cantwell, 1995; Rushing *et al.*1996; Sabbaa-Srur *et al.* 1993). The bacterium *Pseudomonas syringae pv. tomato* synonymously known as *Pseudomonas tomato* causes bacterial speck disease on tomatoes (*Lycopersicon lycopersicum*). In Zimbabwe bacterial speck was one of the four most significant diseases of tomatoes (Dillard *et al.* 1994).

Alternaria alternata (AaP) and *Erwinia* sp. were isolated from pineapple collected from Bidhannagar (Darjeeling), North Bengal and were used for the present studies related to control of postharvest diseases of pineapple. Pineapple is economically important for the production of the fresh and canned fruit. Various diseases have been reported to cause severe losses in pineapple. Other than diseases of the plants the fruits also suffer from post harvest disease problems and that have been considered as one of the constraints for reduced cultivation of pineapple in several areas. Black rot of pineapple is caused by *Chalara paradoxa* is a postharvest disease. Black rot is responsible for high losses on fruits destined to the fresh market and to the processing industry (Wilson *et al.,* 2005). Pink disease is a bacterial infection of the pineapple fruit, characterized by the development of a brown color in the flesh resulting from the processing of fruits infected by *Acetobacter aceti, Erwinia herbicola* and *Gluconobacter oxydans*. The bacteria are brought by insects to the open flowers and under favorable conditions they infect the ovary and reach the flesh of the fruit (Ploetz, 2009, Rohrbach, 1988).

Several bacteria and fungi were isolated from infected fruits and vegetables. The infected fruits and vegetables were collected from different places of North Bengal. Altogether 37 fungi and 76 bacteria were isolated from nine different vegetables and six different fruits. Details of the isolated fungi and bacteria have been shown in table (4.1). Finally, one fungi and one bacterial pathogen of each of the three different fruits/vegetables (Orange, tomato and pineapple) were selected for the present study. After verification of Koch's postulations the fungi were identified in the laboratory and were sent to Indian Type Culture Collection, IARI, New Delhi for confirmation of the identification. The details of the identification results have been presented in materials and methods (Table: 3.3 and 3.5).

The selected fungal pathogens were *Fusarium moniliforme, Alternaria alternata* (AaT) and *Alternaria alternata* (AaP). The selected bacterial pathogens were *Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp.

Shaner *et al.* (1992) stated that plant pathogens exhibit considerable variation in cultural as well as in pathogenic characters. Hence, a thorough knowledge on the morphological and physiological characteristics became necessary after the pathogens were isolated. It also forms the basis of further studies on understanding disease development, host-pathogen interaction and control of the disease caused by the pathogen. Hence it was considered worthwhile to know about the basic morphological and physiological aspects of the fungal pathogen. Therefore, a thorough microscopic observation of the morphological characters of mycelia and spores were performed. All the three fungal pathogens were found to grow and sporulate in PDA medium at room temperature.

Results of microscopic study of the fungi revealed that mycelial mat of *Fusarium moniliforme* (isolated from orange) was white but with maturity, a tinge of pink colour appeared (Plate1:fig. b). The hypha is septate and branched when young. Two different types (macro and micro) of conidia were visible. The micro conidia were born terminally on short hyphae. They were small elliptical or curved 2 celled structures. Macro conidia were formed on mature hypha. Macro conidia were long, curved pointed at the top and with 3 to 4 septa. Rhoobunjongde *et al.* (1991) showed almost similar results of *Fusarium moniliforme*. They found that fungal hyphae were hyaline. Macro conidia were present rarely and their appearance varied from slightly sickle or cigar shaped, 3-4 septa. Micro-conidia were abundantly and variable on size and shape, 0-1 septa. Harrison *et al.* (1990) studied fungal growth of *F. moniliforme* MRC-826 (isolated from Feeds A and B and the lyophilized culture) and was examined after 5 days. Visually, each culture contained pinkish-orange colonies with a tannish-brown underside. Balali and Iranpoor (2005) reported that isolates of *F. oxysporum* from different hosts produced white colonies on PDA with aerial mycelium but the lower surfaces of the colonies were pink or light to dark violet. They also found canoe-shaped macroconidia with 3 to 5 septa and ovoid to ellipsoid uni or bi-cellular, microconidia.

Immature mycelia of *A. alternata* (isolate AaT) were hyaline in colour but on maturity it became gray in colour (Plate:2, fig. b). 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. 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. Our study was supported by similar results (diameter of the mature hypha were 3-5 μm) were also reported by Maiti *et al.* (2007).

Mycelia and conidia of the fungus *A. alternata* (isolate AaP) were shiny black to brown coloured. The length and breadth of the conidia of the fungal isolate ranged between 10-30 μm and 6-12 μm respectively. Mature conidia were several celled with longitudinal and transverse septa (Plate:3,fig.b). Conidia of *Alternaria alternata* formed in natural habitats usually were larger, have longer beaks, and are more uniform in size than those produced *in vitro* on common agar media as reported by Misaghi *et al.* (1978). Slavov *et al.* (2004) reported that The color of fungal (*Alternaria alternata* tobacco pathotype isolate 0-268) colonies was usually dark brown to dark olive green brown, but quite often lighter and almost white colonies sometimes appear under the same conditions with the same medium.

Microscopic study of the bacteria revealed that all the three selected pathogenic bacteria grew in the upper layer and middle layer of the medium. No submerged growth was visible. Hence, all the three bacteria were found to be aerobic. They were catalase positive when grown on nutrient agar media. Our results are in agreement with some other workers (Bonn and Bedford, 1986; Jing- Song et al., 1996; Laby and Beer, 1996).

Pseudomonas syringae was cultured in Pseudomonas Agar media and florescence was observed when the culture was exposed to UV light. Jones (1986) observed a gradient of fluorescence in *Pseudomonas syringae* pv. tomato *(P. s. tomato), P. syringae* pv. *syringae (P. s. syringae),* and *P. viridiflava,* three foliar pathogens of tomato. They also separated the three pathogens by their differential capacity to fluorescence. On king B medium *Pseudomonas* produce yellow fluorescent pigment when seen under UV light.

In the present study, the results of morphological features and biochemical profiles (Section: 4.2 and Table: 4.2, 4.3) of the bacterial isolates suggested that these were motile, glucose fermenting, Gram-negative bacilli and aerobic in nature.

A number of plant species have been reported to possess some natural substances in their leaves and bulb which were toxic to many fungi causing plant diseases. Natural fungi toxic substances are less harmful than chemical fungicides. The plant kingdom therefore has a vast potential for providing antifungal chemicals as only very few compounds have so far been classified and the majority remains to be explored. Sub-Himalayan West Bengal (present study area), has a thick forest cover over large areas and is extremely rich in diverse flora. The region has been declared as hot-spot zone with respect to bio-diversity (Rai and Das, 2002).

Several authors have used plant extracts with antifungal activity to control plant diseases (Al-Howiriny *et al.,* 2005; Bhandary *et al*., 2000; Deena and Thopil, 2000; Ali *et al.,* 2001; Mittal *et al*., 2002; Sharma *et al.,* 2002; Saxena *et al.,* 2003; Saha *et al*., 2005a,b,).

Several plant species with known or unknown bioactivity have been included in the present study. Initially fifty different plant extracts (both aqueous and 50% ethanolic extract) were screened against *F. moniliforme*, *A. alternata (*AaT) and *A. alternata (*AaP) *in vitro* for their antifungal properties by spore germination bioassay technique. Spore germination is a determination factor for the pathogen during the early phase of host colonization. In our study, among the fifty plant extracts tested, *Xanthium strumarium and Datura stramonium* leaf extracts completely inhibited spore germination of *F. moniliforme*, *A. alternata (*AaT) and *A. alternata (*AaP). Leaf extracts of other plants like *Melastoma malabathricum, Borreria alata* and *Mimosa pudica* also significantly inhibited (above 80%) spore germination of the pathogen as shown in the results presented in the experimental (Table: 4.6).

In our study poisoned food assay was performed with two (*Xanthium strumarium* and *Datura stramonium*) selected plant extracts to further confirm their fungi toxic characters *in vitro.* Ethanol and aqueous leaf extract of two plants ((*Xanthium strumarium* and *Datura stramonium*) were also tested for their efficacy by poisoned food technique. From the results (Table:4.5) it was clear that aqueous and 50% ethanolic leaf extracts of *Xanthium strumarium* showed significant inhibitory effect against *F. moniliforme* with 82.22% and 85.55% inhibition respectively in comparison to control plates. Leaf extract of *Datura stramonium* showed less inhibitory activity than *Xanthium strumarium* leaf

extract as evident from the results (Table: 4.7). *Datura stramonium* and *Xanthium strumarium* aqueous leaf extract also showed more than 50% inhibition of growth of the fungus *A. alternata* (AaT) in comparison to growth observed in control plates. *A. alternata* (AaP) was also controlled by the leaf extracts (aqueous and 50% ethanolic) of both the plants as mentioned (Table: 4.7).

Several other studies supported that *Xanthium strumarium* leaf extracts have antifungal activity. Damayanti *et al.,* (1996) treated pineapple fruits infested with *C. paradoxa* by *X. strumarium* extract and found reduced disease severity. *Xanthium strumarium* was the most effective followed by *Allium sativum and* ethanol was suitable for extraction of the inhibitory substance from *X. strumarium.* Acetonitrile was highly toxic to this fungus. Millipore filter-sterilized extracts had a more inhibitory effect on the fungus than the autoclaved samples. Floral malformation caused by *Fusarium mangiferae* is a serious threat to mango cultivation in various countries. Methanol-water (70/30 v/v) extracts of *Datura stramonium* showed strong antifungal activity against the said pathogen (Usha *et al.*, 2009). Many tropical medicinal plants and species have been used as pest control agents (Lale, 1992). Peasant farmers and researchers often claim successful use of plant materials in insect pest control including vegetable oils (Sahayaraj, 2008) and powders of plant parts (Lajide *et al.*, 1998). Numerous studies have documented about the antifungal (Suhr and Nielson, 2003; Mishra and Dubey, 1994) and antibacterial (Canillac and Mourey, 2001) effect of plant essential oils. Examination of indigenous local herbs and plant materials have also been reported from around the world as evidenced from the articles from India (Ahmad and Beg, 2001), Australia (Cox *et al.*, 1998), Argentina (Penna *et al.*, 2001) and Finland (Rauha *et al.*, 2000). Higher plants contain a wide spectrum of secondary metabolites such as phenols, flavonoids, quinones, tannins, essential oils, alkaloids, saponins and sterols which showed antimicrobial activity.

Jha and Sharma (2008) screened leaf extracts of 83 plant species (aqueous and autoclaved) *in vitro* against *Rhizoctonia bataticola.* A few plant species (*Rannunculus scleratus, Xanthium strumarium, Ipomoea carnea, Ocimum basilicum* and *Eclipta alba)* showed antifungal activities. The extract of *R. scleretus* was highly

effective. Among the extracts *X. strumarium*, *O. basilicum* and *I. carnea*, had their effects on sclerotia.

Khanzada (2006) showed the effectiveness of various extracts against *C. paradoxa.* Effectiveness was in the decreasing order of *Meriandra bengalensis, Mentha piperita, Curcuma longa, Phlogacanthus thyrsiflorus, Toona ciliata, Vitex negundo, Azadirachta indica, Eupatorium birmanicum, Ocimum sanctum and Leucas aspera.* The *D. alba* and *C. procera* were found effective @ 1.5 and 2% followed by *C. sativus* (2%) in reducing the mycelial growth of the fungus over the non amended control. Sclerotial production was inhibited by the leaf extracts of *D. alba* and *C. procera* at the concentration of 2% the growth.

On the basis of the results of the present study (Table: 4.6 and 4.7), *Xanthium strumarium* and *Datura stramonium* were selected for agar cup bioassay and for determination of their MIC (minimum inhibitory concentration). MIC was determined for two different extracts (crude extracts as stated above and bioactive fraction of the leaf extracts) of the two selected plants (Table: 4.8 to 4.11). Crude extract used throughout the study are the aqueous leaf extracts. Bioactive fractions were found following extraction technique as stated in the materials methods (section: 3.5.4.2). From the results(Table: 4.9) it was found that minimum inhibitory concentration (MIC) of bioactive fraction of *Xanthium strumarium* leaf extracts *against F. moniliforme, A. alternata* (AaT) and *A. alternata* (AaP) were 100 µg/ml ;200 µg/ml ; 500µg/ml respectively.

Our results seem to be very similar to that of Kanauchi *et al.*, (1999). They showed that the extract of cocklebur (*X. strumarium)* contains xanthatin, an antibacterial substance. Minimum inhibitory concentrations (MICs) of xanthatin were 12.5-100µg/ml against *Bacillus* sp. MICs of xanthatin were 25-100 µg/ml against *Candida* sp., *Pichia* sp., *Saccharomycopsis* sp. and *Torulaspora* sp. They also suggested that xanthatin from cocklebur leaf extract against *Bacillus* sp. and some film-forming yeast may be used to prevent contamination in koji and during production of alcoholic beverages.

From the results (Table 4.10 and 4.11) of the present study it was evident that MIC values of *Datura stramonium leaf extracts against F. moniliforme, A. alternata* (AaT) and *A. alternata* (AaP) 0.50mg/ml & 200µg/ml ; 2.00mg/ml & 300 µg/ml ;

5.00mg/ml & 550µg/ml (respectively for aqueous extract and bioactive fraction of *D. stramonium*).

Maja and Botha (2008) reported antibacterial activity of Plant material extracts of *D. angustifolia; D. stramonium* and *Z. capense* against *T. forsythensis*. They also reported that the MIC value of the extracts were within the range of 0.01-10mg ml^{-1}. Ahmed *et al.*, (2005) screened antifungal compounds (terpenoids) from the roots of the wild carrot, *Daucus carota* L. ssp. *carota* (Apiaceae) and found to contain a range of antifungal activity against *Fusarium oxysporum* and *Aspergillus niger*. Scher *et al.* (2004) prepared a dichloromethane and a methanol extract of the liverwort *Bazzania trilobata* (L.) S.F. Gray (Lepidoziaceae) and showed their antifungal activity against the phytopathogenic fungi *Botrytis cinerea*, *Cladosporium cucumerinum*, *Phythophthora infestans*, *Pyricularia oryzae* and *Septoria tritici* due to the presence of terpenoids. From these extracts, Scher *et al.* (2004) isolated six antifungal sesquiterpenes: 5- and 7-hydroxycalamenene, drimenol, drimenal, viridiflorol, gymnomitrol and chloroisopiagiochin. Fujita *et al.*, (2005) isolated D. Polygodial, a sesquiterpene from *Polygonum punctatum* Elliot. (Polygonaceae) and found fungicidal activity against a food spoilage yeast, *Zygosaccharomyces bailii*. In the search for new sources of sesquiterpene lactones, Barrero *et al.*, (*2000*) investigated antifungal efficacy of sesquiterpene lactones isolated from the six *Centaurea* species (*C. bombycina* Boiss ex D.C., *C. granatensis* Boiss, *C. monticola* Boiss, *C. incana* Desf., *C. maroccana* Ball. and *C. sulphurea* Willd.) and activity of extracts of the plants were tested against the fungus *Cunninghamella echinulata*. They also reported that costunolide and dehydrocostunolide, two compounds were responsible for the antifungal activity. Skaltsa *et al.*, (2004) reported some other antifungal compounds (4-*epi*-sonchucarpolide and their 8-(3-hydroxy-4-acetoxy-2-methylene-butanoyloxy) from other *Centaurea* species. (Marthanda *et al* 2005) reported that the diterpenoids 16α-hydroxy-cleroda-3,13-(14)-*Z*-diene-15,16-olide and 16-oxo-cleroda-3,13-(14)-*E*-diene-15-oic acid isolated from the hexane extract of the seeds of *Polyalthia longifolia* (Som.) Thw (Annonaceae) exhibited antifungal activity. The antifungal terpenoids from medicinal species also included diterpenoids and triterpenoids. Some of these compounds were isolated by bioassay-guided fractionation after previously detecting antifungal activity on the part of the plant. Bioassay-guided fractionation of the

methanol and ethyl acetate extracts of two lianas from the genus *Casimirella* (Miers) RA Howard (Icacinaceae) collected in the Suriname rainforest, has led to the isolation of five new diterpenoids: humirianthone, 1-hydroxy-humirianthone, 15*R*humirianthol, patagonol and patagonal. All the diterpenoids showed activity against phytopathogenic fungi (Adou *et al.*, 2005).

Fungicides are a cheap and quick method to control pathogen of several diseases because of its direct interaction and antagonist mode of action against the pathogens. It either kills the pathogen instantly or minimizes the growth and proliferation of pathogen immediately. In the present study, minimum inhibitory concentration (MIC) of Bavistin and Roko were determined against the three selected fungal pathogens [*F. moniliforme, A .alternata*(AaT) and *A. alternata* (AaP)] of the present study. The objective of use of fungicides in the present study, are to compare the fungicides with that of botanicals. MIC values of Bavistin and Roko were 1.00 mg/ml, 3.00 mg/ml respectively against *F. moniliforme*. MIC values of Bavistin and Roko against *A. alternata* (AaT) were 3.00 mg/ml, 5.00 mg/ml and the same against *A. alternata* (AaP) were 8.00 mg/ml, 10.00 mg/ml respectively. The details of the results have been presented in the experimental section (Table: 4.12 and 4.13).

Fungicides have been used by several workers. Cabanas *et al.*, (2009) observed that 69·4% of the strains isolated from apples and pears were resistant to TBZ. Sensitive isolates were inhibited at 0·25–0·5 $\mu g\ ml^{-1}$ whilst resistant isolates still grew at 512 $\mu g\ ml^{-1}$. Dipping in 1000 ppm TBZ reduced decay caused by *Penicillium digitatum* and *P. italicum*. Doses of 500 and 200 ppm were optimum for maintaining green buttons on Kinnow mandarins and lemons, respectively. Changes in ascorbic acid, acidity, and reducing, non-reducing and total sugars were insignificant.

An antifungal antibiotic derived from a local strain of *Bacillus subtilis* (AECL-69) controlled *Alternaria citri* during storage. No residual antibiotic activity was found in peel or juice in Kinnow mandarins and Valencia oranges after storage at 4-5°C for 3 and 4 months, respectively (Babu and Reddy, 1986).

From the results it was concluded that the two fungicides (Bavistin and Roko) were effective only at higher concentrations if compared with botanicals extracted from *Xanthium strumarium* and *Datura stramonium*.

The antifungal activity of aqueous, petroleum ether, benzene, chloroform, methanol and ethanol extracts and alkaloid extract of *Prosopis juliflora* (Sw.) DC. Leaves (Mimosaceae) were evaluated for antifungal activity by poisoned food technique against *Alternaria alternata* a causal organism of brown spot of tobacco. Aqueous extract recorded highly significant antifungal activity at 24% concentration. Among different solvent extracts tested, methanol and ethanol extract recorded highly significant antifungal activity. Methanol extract was further subjected to fractionation guided by antifungal activity leading to the isolation of alkaloid extract, which was also recorded highly significant antifungal activity against the test fungus and the minimum inhibitory activity was recorded at 1000 ppm. The antifungal activity of alkaloid extract was compared with synthetic fungicides viz., blitox, captan, dithane M-45 and thiram at their recommended dosage of 2000 ppm indicating that the alkaloid extract was highly effective even at the dosage lesser than the synthetic fungicides (Raghavendra, 2009).

P18 displayed potent fungicidal activity (MIC: 12.5~25 μM) against pathogenic fungi, *Candida albicans, Trichosporon beigelii, Aspergillus flavus* and *Fusarium oxysporum* (Lee *et al.*, 2004).

Singh *et al.,* (1980) showed that some essential oils (extracted from *Cymbopogon martinii, C. oliveri, C. sp.,*and *T. ammi*) were more active than some of the prevalent synthetic fungicides and thus they suggested exploitation of natural fungicides following successful infield trials.

Christian and Susana(2008) showed that the essential oils of 18 plants possess antifungal properties against three common corn pathogens: *Penicillium, Fusarium,* and *Pythium.* Five oils [cinnamon (*Cinnamomum zeylanicum* Blume), clove (*Eugenia caryophyllata* Thunb.), oregano (*Origanum minutiflorum* O. Schwarz and P.H. Davis), savory (*Satureja montana* L.), and thyme (*Thymus vulgaris* L.)] completely controlled all three pathogens *in vitro.* The minimum inhibitory concentration (MIC) for all pathogens was 800 μL L^{-1} and seedlings presented no phytotoxicity symptoms in the germination test at rates up to 64 μL kg^{-1} active ingredient (MIC x 20).

In the present study the MIC values of *Xanthium strumarium, Datura stramonium* plant leaf extract (aqueous and bioactive fraction) against *Xanthomonas* sp., *Pseudomonas syringae* and *Erwinia* sp. were performed. From the results it was evident

that MIC values of *Xanthium strumarium* leaf extracts (crude and bioactive fraction) *against Xanthomonas sp, Pseudomonas sp* and *Erwinia* sp. were 0.75mg/ml & 50µg/ml; 1.00mg/ml & 125µg/ml; 1.00mg/ml & 100µg/ml, respectively. Similarly MIC values of *Datura stramonium* leaf extracts (aqueous and bioactive fraction) *against Xanthomonas sp, Pseudomonas sp* and *Erwinia sp* were 0.50mg/ml & 50µg/ml ; 1.00mg/ml & 100 µg/ml; 0.75mg/ml & 75 µg/ml respectively.

Several other studies have also indicated the potential of plant extracts in the control of diseases caused by *X. campestris* in several important crop plants. Akhtar *et al.*, (1995) tested about 208 diffusates from various plants such as forest trees, shrubs, herbs, fruit seeds etc. against *Xanthomonas campestris* Pv. *citri.* and diffusates from various parts of *Phyllanthus emblica, Acacia nilotica, Sapindus mukorossis* and *Terminalia chebula* exhibited an inhibition zone 4.83-6mm at 50 g/liter appeared to be the most effective. Satish *et al.*, (1999) found inhibitory effect of extracts of extracts from *Acacia arabica, Achras zapota* and from other 6 higher plants against various pathovars of *Xanthomonas campestris*. Csizinszky *et al.*, (1993) also reported that *Chamomilla recutita* and *Chamaemelum nobile* extracts inhibited the growth of *Xanthomonas campestris* Pv. *citri* strains causing citrus bacterial canker disease. Patil and Ghoderao (1997) also evaluated some medicinal and aromatic plants against cotton bacterial blight infection and *Azadirachta indica* and *Ipomoea carnea* extracts were found effective at reducing the incidence and intensity of the disease. Root and leaf extracts of *Adhatoda zeylanica* showed *in vitro* inhibition of *Xanthomonas campestris* Pv. *vignicola* by producing inhibition zones of 1.35 cm. and 1.52 cm respectively (Thammaiah *et al.*, 1995).

Sixteen plant extracts exhibited good anti-*H. pylori* activity out of twenty plant extracts analyzed by Nariman (2009) following disc diffusion method. Ten most active extracts were *Carum bulbocastanum, Carum carvi, Mentha longifolia, Saliva limbata, Saliva sclarea, Ziziphora clinopodioides, Thymus caramanicus, Glycyrrhiza glabra, Xanthium brasilicum* and *Trachyspermum copticum.* MIC of the 10 biologically active plant extracts was within the range of 31 to 500 µg/ml.

Eftekhar *et al.*, (2005) investigated the antibacterial activity of the methanol extracts of the aerial parts of the *Datura innoxia* and *Datura stramonium*. The extracts

showed activity against Gram (+) bacteria in a dose dependent manner. Little or no antibacterial activity was found against *Escherichia coli* and *Pseudomonas aeruginosa.*

In order to separate antifungal compounds in the potential plant extracts, the extracts are subjected to "on the chromatogram bio assay" on the thin layer chromatography. The method is one of the very quick and easy methods for screening antifungal compounds in phytoextracts (Guleria and Kumar, 2000). Several workers (Kagale *et al.,* 2004 and Saha *et al.*, 2005a) have utilized the method to isolate natural products of various chemical compounds.

During the present study (Table: 4.18) aqueous *Xanthium strumarium* leaf extract was developed on TLC plates and sprayed with spores of *Fusarium moniliforme, Alternaria alternata* (isolate AaT) and *Alternaria alternata* (AaP). R_f of antifungal zones created by the crude leaf extract was 0.68 against both *Fusarium moniliforme,* and both *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, bioactive fraction of *Xanthium strumarium* leaf extracts showed three antifungal zones at R_f-0.42; R_f -0.17 and 0.72 altogether against the pathogens tested (Table- 4.19; plate 15: fig. A; plate 16: fig. A; plate 17: fig. a).

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 R_f- 0.81. Partially purified bioactive fraction of *Datura stramonium* leaf extracts showed three antifungal zones 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 (R_f- 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).

Reddy *et al.* (2007) reported the antifungal component of cloves. They isolated, characterized and tested the efficacy of cloves against *Aspergillus* spp. The major

component, eugenol was identified on TLC plate as dark coloured spot with R_f 0.5 along with standard. In TLC plate bioautography test, TLC plates were spray inoculated with four species of *Aspergillus* (*A. flavus*, *A. paraciticus*, *A.niger*, *A. ochraceus*) and eugenol on TLC plates inhibited mycelia growth of all four species of *Aspergillus*.

Structural identification of the antifungal compound was made with the help of various spectroscopic analyses.

In the course of the present study, the fruits were subjected to pathogenicity test to find most susceptible fruits. Plant extracts were sprayed on the most susceptible fruits and then the fruits were challenge inoculated with their pathogens separately. From the results it was evident that the plant extracts significantly controlled the post harvest diseases of the fruits (Table 4.22 to 4.27).

Use of chemical fungicides is the concern in modern society in terms of human toxicity and hazardous effects on natural environments (Goto, 1990). Biological control provides an alternative where a micro-organism that is non pathogenic to the plant but antagonist towards plant pathogen is used. In cultivation where higher production is all of the matter, post harvest disease requires intense management and planning. Post harvest disease can be controlled by spraying exogenous fungicides but due to awareness on harmful effect on fungicides of environment as well as humans, it's essential to use eco-friendly measures. Chemical fungicides also adversely affect the microbial population present in the ecosystem. Hence, Search for effective bio-control agents for the management of plant diseases has been intensified in recent years to reduce the dependence on chemical fungicides (Droby *et al.*, 1992). Moreover, the post-harvest phase is suited to the application of biological control methods (Mari and Guizzardi, 1998). Although biological control of many pathogens are reported in literature, but works to control post harvest disease of orange, tomato and pineapple by antagonistic microbes are scanty.

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., *Pseudomonas syringae* and *Erwinia* sp.). Fungal antagonist

(*Trichoderma harzianum* and *Aspergillus flavus*) and bacterial isolates (*Lysinibacillus sphaericus* and *Pseudomonas* sp.) were isolated from soil and selected through dual culture for the present study in controlling post harvest pathogens(Section 4 : 11) , *Trichoderma harzianum* and *Aspergillus flavus* inhibited *Fusarium moniliforme*, *Alernaria alternata* (AaT) and *Alernaria alternata* (AaP) in dual cultures. Results of dual cultures involving SB1 or SB2 as one of the cultures against the pathogens (separately) clearly indicated that SB2 is the best biocontrol agent between the two bacterial antagonists. SB2 inhibited (88.57%) growth of *Xanthomonas* sp. SB1 inhibited (77.14%) growth inhibition over *Xanthomonas* sp. SB1 also inhibited growth (61.11%) of *Alternaria alternata* (AaP). *Alternaria alternata*(AaT) was least inhibited by SF1. *Trichoderma harzianum* showed highest inhibition (90.00%) over *Alternaria alternata*(AaP). Results of the dual culture involving the four antagonists clearly indicated that SF1 is the best biocontrol agent.

Among fungal antagonists, *Trichoderma* sp. is most commonly used, mainly due to their high efficacy in controlling several diseases. Several authors have reported the successful use of different isolates of *Trichoderma* for controlling many plant diseases (Jadeja, 2003; Roberts *et al.*, 2005). *Bacillus* sp. also has been used by several workers for control of plant pathogens. Meena *et al.*, (2000) controlled *Phomopsis vexans* by using *Bacillus* sp. The culture filtrates of *A. flavus* and of the *Trichoderma harzianum* was used *in vitro* and the results were encouraging (Elad, 2000; Perello *et al.*, 2006)

In the present study, cell-free culture filtrate *of Trichoderma harzianum* and *Aspergillus flavus* significantly inhibited *Fusarium moniliforme*, *Alernaria alternata* (isolate AaT) and *Alernaria alternata* (isolate AaP) in poisoned food technique. In dual culture antagonists directly inhibited the pathogen but in case of poisoned food experiment cell-free culture filtrates showed inhibition (Section 4:13). It clearly indicated that the inhibiting activity lies in the extracellular fluid.

Cell free culture filtrates have been used to demonstrate the rate of antibiosis, a mechanism of biological control (Khara and Hadwan, 1990; Tu, 1992). Shanmugam and Varma (1999) clearly established the efficacy of the antagonists *Aspergillus niger*, *A. fumigatus*, *A. flavus* and *Trichoderma viride* in inhibiting pathogen. Thus our results are in agreement with that of earlier workers.

Naturforsch, (2002) reported that the crude MeOH extract from the dual culture of *T. harzianum* and *C. roseus* callus showed a very strong antimicrobial activity against the Grampositive bacteria *Staphylococcus aureus* and *Bacillus subtilis,* with an MIC of 31.3 µg/ml for both. They also reported moderate inhibitory activity of the isolated compounds against the yeast *Saccharomyces cerevisiae*, with an MIC of 500µg/ml. They also tried to isolate the active compound under the guidance of MIC assay.

In this study cell free culture filtrates were subjected to six biochemical tests (Pectinase, phosphatase, cellulase, chitinase, DNase activity and siderophore production) to know their mechanism of antimicrobial activity. The results have been presented in experimental section: 4.14. (Table: 4.32 & 4.33). *Aspergillus flavus*, *Trichoderma harzianum, Lysinibacillus sphericus* and *Pseudomonas* sp did *not* show pectinase and phosphatase activity. All the four organisms mentioned above exhibited cellulase activity. *Aspergillus flavus*, *Lysinibacillus sphaericus* and *Pseudomonas* sp. produced siderophore too. Out of four organisms only *Trichoderma harzianum* does not produce siderophore. Chitinase activity was shown by lone *Trichoderma harzianum* among the four organisms tested. Lipase activity test was performed in case of the two antagonistic bacteria only and both the bacteria produced lipase.

Trichoderma harzianum is a known producer of cellulolytic and chitinolytic enzymes that are extensively used for the degradation of cellulose and chitin materials particularly in textile and paper industries, besides its use in wastewater treatment (Ahmed *et al.*, 2007). Several biocontrol agents alleviate the growth of pathogenic fungi by producing extracellular chitinase, which degrades the chitin polymers of fungal cell wall (Mathivanan *et al.*, 1998; Mathivanan *et al.*, 2000). Growth inhibition of the pathogens by the *Trichoderma* metabolites were reported (Ghisalberti and Sivasithamparam, 1991). The phenomenon and related mechanisms have been explained by many authors (Lynch, 1990; Inbar, *et al.*, 1994; Papavizas, 1985). Belanger *et al.* (1995) reported that *T. harzianum* antagonize first by antibiosis (leading to cell death) and then degrade cell wall by chitinolytic enzymes.

Most species of *Aspergillus* are known to produce several hydroxamate-type siderophores and many reports on the isolation and characterization of siderophores have been published (Dube *et al.*, 2000). Basha and Ulaganathan (2002) reported that *Bacillus*

sp (strain BC121, sorghum rhizosphere isolate) showed high antagonistic activity against *Curvularia lunata*. In dual cultures, the *Bacillus* strain BC121 inhibited the *C. lunata* up to 60% in terms of dry weight. This strain also produced a clear halo region on chitin agar medium plates containing 0.5% colloidal chitin, indicating chitinase activity. Mikani *et al.,* (2008) screened some potential *Pseudomonas* spp. strains for their antifungal activities against some pathogens. Chaiharn, (2009) demonstrated antagonistic activity towards the rice pathogens by potential biocontrol agents (of soil of in Thailand and other countries).

In the present study degradation of hyphae were prominent when interacting zones were seen under scanning electron microscope (SEM). The results of SEM studies have been presented in the following section.

Finally, SEM analysis of pathogen and antagonistic bacteria and fungi were performed. It has been seen from the results of SEM analysis that spores of antagonistic fungi *A. flavus* (SF7) attached with the pathogenic hyphae and degraded the hyphae by releasing certain chemicals. On the other hand the antagonistic bacteria *Pseudomonas* sp. (SB2) colonized the hyphae of some of the pathogens tested indicating their affinity towards the hyphae of the said pathogen. In densely colonized (by the antagonistic bacteria) pathogenic hyphae scars of damage were prominent. This study indicated that the bacteria may have produced certain fungal cell wall degrading enzyme which in turn degraded the hyphae. SEM analysis also showed that bacteria were occupying the degraded hyphae. As degraded portion showed cell aggregates of the antagonistic bacteria.

The macroscopic and microscopic (SEM) observations, of plates with the pathogens cultured with filtrates of *Trichoderma* spp. suggested that *Trichoderma* spp. were able to induce morphological alterations in both *Aspergillus flavus* and *Fusarium moniliforme (*Calistru *et al.*, 1997*)*. *Bacillus* strain BC121 inhibited the *C. lunata* and Scanning electron microscopic observations showed a clear hyphal lysis and degradation of fungal cell wall. (Basha and Ulaganathan, 2002). Our study also suggested hyphal lysis, degradation and morphological changes. Thus our study is in agreement with the previous study.

During this study, certain new facts of fundamental importance have been revealed. All other results of the present study are in conformity with those obtained by previous workers. Thus all the investigations have confirmed and extended some of the findings of the earlier workers. This work dealt with isolation and identification of pathogens of the three major fruits/vegetables prevalent in north Bengal. The results of *in vivo* studies would definitely help in designing some bioformulations and applicable phytoextracts for control of the post harvest diseases in pineapple, orange and tomato, the three economically important horticultural crops of north Bengal. Although two fungal organisms (*Trichoderma harzianum* and *Aspergillus flavus)* showed antifungal efficacy but *Aspergillus flavus* is not suggested for its mycotoxin producing activity. It is an opportunistic pathogen causing invasive and non-invasive aspergillosis in humans, animals, and insects. It also causes allergic reactions in humans (Hedayati, 2007; Yu *et al.* 2005).The studies involving *Aspergillus flavus* have enlightened us to understand the mechanism of activity of antibiosis. Two bacterial soil isolates (*Lysinibacillus sphaericus* and *Pseudomonas* sp.) may also be exploited for controlling post harvest pathogens. Two plant extracts have also been selected for control of the post harvest pathogens of the fruits and vegetables tested. Leaf extracts of *Xanthium strumarium* and *Datura stramonium* have shown potential antifungal activity both *in vitro* and *in vivo.* These may be utilized for production of bioformulations applicable to the harvested fruits.