POST HARVEST DISEASES OF SOME SELECTED SALAD VEGETABLES, THEIR CAUSES AND MANAGEMENT

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POST HARVEST DISEASES OF SOME SELECTED SALAD VEGETABLES, THEIR CAUSES AND MANAGEMENT

BY

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CERTIFICATE

This is to certify that the thesis entitled **"POST HARVEST DISEASES OF SOME SELECTED SALAD VEGETABLES, THEIR CAUSES AND MANAGEMENT"** submitted to the **DEPARTMENT OF PLANT PATHOLOGY**, Sher-e-Bangla Agricultural University, Dhaka in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE (M.S.) IN PLANT PATHOLOGY**, embodies the results of a piece of bona fide research work carried out by **SADIA SHARMIN** bearing **REGISTRATION NO. 13-05447**, under my supervision and guidance. No part of this thesis has been submitted for any other degree or diploma in any other institution.

I further certify that any help or sources of information received during the course of this investigation have been duly acknowledged.

Dated: 28.12.2020
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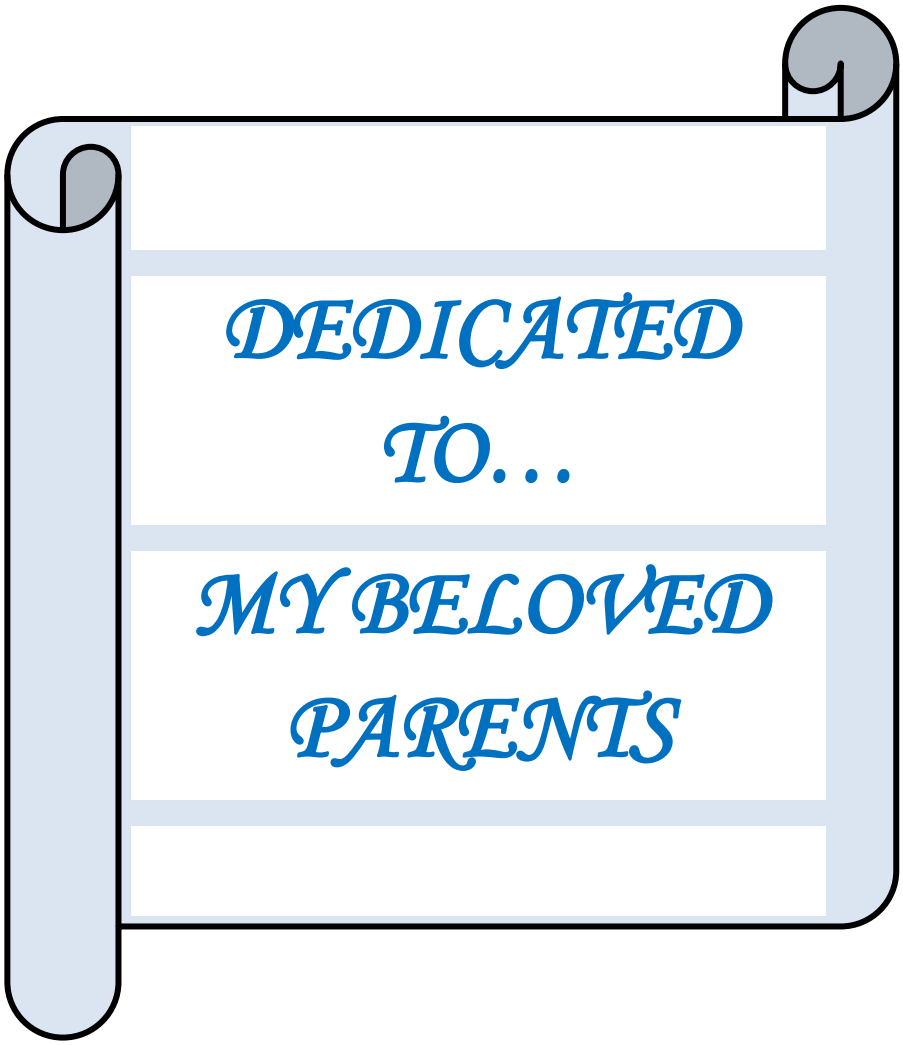
DEDICATED
TO…
MY BELOVED
PARENTS

LIST OF SYMBOLS AND ABBREVIATIONS

No = Number

% = Percentage

et al. = And others

^{0}C = Degree Celsius

@ = at the rate

J = Journal

ppm = parts per million

Kg = Kilogram

g = Gram

ml = Mililiter

cm = Centimeter

SAU = Sher-e-Bangla Agricultural university

NA = Nutrient Agar (media)

PDA = Potato Dextrose Agar (media)

SIM = Sulphide Indole Motility (media)

Cfu = Colony forming unit

BLAST= Basic Local Alignment Search Tool

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The Author

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POST HARVEST DISEASES OF SOME SELECTED SALAD VEGETABLES, THEIR CAUSES AND MANAGEMENT

ABSTRACT

A study was conducted to observe the post harvest diseases of some selected salad vegetables and to identify their causes. The experiment was conducted in the MS Laboratory of Department of the Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University during the period from March, 2019 to March, 2020. Samples were collected from local markets of Dhaka city. In this study, seven different fungal species and four different bacterial strains were isolated. Aspergillus rot (*Aspergillus niger* and *Aspergillus flavus*), cottony soft rot (*Sclerotinia sclerotiorum*), fusarium rot (*Fusarium* sp), sour rot (*Geotrichum candidum),* anthracnose (*Colletotrichum capsici*), green mold (*Trichoderma* sp) and bacterial soft rot (*Serratia marcescens*, *Bacillus* sp and *Pseudomonas* sp) diseases were identified. Fungi were isolated by growing them in moist chamber and PDA medium. Bacteria were isolated by dilution plate method and growing them in NA medium by spread plate method and streak plate method. Bacteria were identified by studying the morphological and cultural characteristics and performing several biochemical tests like Gram-staining test, KOH solubility test, oxidase test, catalase test, motility test, citrate utilization test, casein hydrolysis test, lactose fermentation test, sucrose fermentation test, dextrose fermentation test. Bacillus agar and citrimide agar medium were used to separate *Bacillus* and *Pseudomonas,* respectively. Pathogenicity test of some pathogens were also performed. Identification of the bacteria from cucumber was further confirmed by extracting DNA from the bacterial cultures. The samples were subjected to PCR using 27F and 1492R primers. The D1/D2 of 16S rDNA sequences of *Serratia marcescens* isolates were submitted to NCBI for deposition in the GenBank and accession number. The provided accession number MW404210 for *Serratia marcescens* strain SAU-1. Effects of different storage containers on different vegetables and their microbial growth were also observed.

Chapter I

INTRODUCTION

Bangladesh is mainly agro based country. Many types of crops are grown in this country. Salad vegetables are one of them. A mixture of raw usually green leafy vegetables combined with other vegetables and served with a dressing is called salad. Due to high respiration and transpiration rate, salad vegetables deteriorate very rapidly. Salad vegetables is very fleshy, it is another point of deterioration. Salad vegetables contain various vitamins, calcium, folic acid and fiber. Salads are rich in antioxidants and it protects our body from the deadly cancer cells. Tomato, cucumber, carrot and capsicum are becoming important salad vegetables in the whole world. They deteriorate rapidly during handling and storage time. Salad vegetables are affected by different post harvest diseases. The diseases which develop on harvested parts of the plants like fruits, seeds and tubers are called the post harvest diseases.

Tomato (*Solanum lycopersicum*) is an hervaceous plant that belongs to the family solanaceae, a nightshade family that is widely cultivated for fruits throughout the humid and subtropics (Dania and Okoye, 2017). Tomato production in the whole world is 18,22,56,458 tonnes and tomato production in Bangladesh is 3,85,038 tonnes (FAO, 2018). 95% water, 4% carbohydrates and less than 1% each of fat and protein contains in a tomato (Wikipedia). Tomato has a high customers acknowledgement and request because of its adequate and well balanced nutrition containing minerals like potassium, zinc, magnesium etc. (Osemwegie *et al.*, 2010) and plentiful in vitamins A, B, C, and E (John *et al.*, 2010; Uke and Cheijina, 2012) which assists with limiting the chance of prostate and breast cancer (Giovannucci, 1999). Tomato is susceptible to many destructive plant microbes particularly more inclined to postharvest fungal pathogens (Taskeen-Un-Nisha *et al.*, 2011). Harvested fruits of tomato convey propagules of microorganisms which become dormant in the field and cause spoiling of tomato fruits when condition got favorable during storage and so proper handling is important to limiting the frequency of post harvest decay

(Mahovic *et al.,* 2004). Gray mold, rhizopus rot, sour rot, canker, anthracnose, buckey rot, fusarium rot, phoma rot, southern blight, early blight, bacterial soft rot, late blight, bacterial canker are the post harvest diseases of tomato.

The carrot (*Daucus carota* subsp. *sativus*) usually orange in color is a root crop. They are originated from Europe and Southern Asia. Carrot production in whole world is 3,99,96,287 tonnes (FAO, 2018). Carrot is a good source of manganese and magnesium which needed for bone and protein, creating new cells, activating vitamin B, nerves, ergonomic muscles, blood clotting, energy production (Tucker *et al.,*1999). Raw carrots supply 88% water, 9% carbohydrate, 0.9% protein, 2.8% dietary fiber, 1% ash and 0.2% fat (Wikipedia). Several types of carrot cultivars are grown today and different cultures have particular preferences for the shapes and root colors of carrots (Rubatsky, 2002). Farm procured a huge amount of benefit from cultivating carrots since it requires fewer amounts of input, plant security measures and good quality of water (Ahmad *et al*., 2005). High percentage of carrot fruits are lost annually because of post harvest rotting caused by fungal pathogen (Mahale *et al.,* 2008). Sour rot, crown rot, cottony soft rot, phytophthora root rot, black rot, root dieback and bacterial soft rot are the post harvest disease of carrot. The main microorganism belonging to the black root rot as the significant contributors of the disease are *Fusarium oxysporum, Fusarium solani, Pestalotia longiseta* and others (Manici *et al.*, 2005).

Cucumber (*Cucumis sativus*) is belongs to the gourd family, cucurbitaceae. It is a creeping vine and it bears cucumiform fruits. The cucumber is originated in South Asia, but now grows most of the countries. Cucumber production in whole world is 7,52,19,440 tonnes and cucumber production in Bangladesh is 1,10,219 tonnes (FAO, 2018). In a 100 gram raw cucumber with peel, 95% water, 16 kilocalories and 16% vitamin K are remaining (Wikipedia). Anthracnose, gummy stem blight, black spot, choanephora wet rot, fruit rot, belly rot, diplodia fruit rot, curvularia fruit rot, aspergillus fruit rot, bacterial soft rot, bacterial fruit rot are the post harvest diseases of cucumber. Cucumber

plants are subjected to several fungal diseases that affect the yield quantity and quality including *Alternaria tenuis, Alternaria alternata, Botryrtis cinerea, Choanephora cucurbitarum, Didymella bryoniae, Fusarium oxysporum, Geotrichum candidum, Penicillium oxalicum, Phytophthora capsici, Rhizopus rigricans* (Blancard *et al.,*2005; Farrag *et al.,* 2007; Sani *et al.,* 2015; Ziedan and Saad, 2016).

Capsicum (*Capsicum annum*) the bell pepper, belongs to the nightshade family, solanaceae. It is originated from America. Bell pepper production in whole world is 7,32,524 tonnes (FAO, 2018). The bell pepper is an economically important crop for both local and export market (Shehata *et al.,* 2013). It is a good source of vitamin A and vitamin C as well as phenolic compounds and it is also known for its antioxidant properties (Shotorbani *et al.,* 2013). It is believed to prevent certain types of cardio vascular disease, atherosclerosis, cancer and haemorrhage with its nutritional contribution (Marin *et al.,* 2004). Bell peppers contain 94% water, 5% carbohydrates and negligible fat and protein (Wikipedia). During delayed storage, the main factors for the quality degradation of sweet pepper include poor outer appearance, rot development, shrinking related water loss and its high vulnerability to chilling injury (Shehata *et al.,* 2013). Gray mold, anthracnose, fruit rot, late blight, bacterial soft rot are the post harvest diseases of capsicum.

Microbial fruit infections could occur during crop cultivation, harvesting, post harvest handling at processing, storage, transportation and packing and distribution (loading and offloading) at various channels and selling outlets of which bacteria and fungi are prevalence (Akinyele and akinkunmi, 2012; Barth *et al.,* 2009; Fung, 2009).

Handler and consumer therefore attaches a lot of important to the retention of fruit green colour, firmness, as quality attributes during handling and storage (Sigge *et al.,* 2001). These quality parameters are functions of temperature, relative humidity (Gorini *et al,* 1977) and air composition of the handling on

storage environment. Recent advance in plant pathology, various innovative techniques are established to manage post harvest diseases.

OBJECTIVES:

Keeping these facts in mind the study will be undertaken to achieve the following objectives:

1. To isolate, identify and characterize the causal agents of selected salad vegetables.
2. To characterize the bacteria through molecular method.
3. To determine the effect of different storage containers on post harvest deterioration of salad vegetables.

Chapter II

REVIEW OF LITERATURE

2.1. Isolation and identification of postharvest diseases of tomato

Rodrigues and Kakde (2019) worked with tomato fruits and they isolated *Alternaria alternata, Aspergillus niger, Aspergillus flavus, Colletotrichum* sp, *Rhizopus* sp, *Fusarium oxysporum, Botrytis cinerea, Penicillium digitatum, Penicillium chrysogenum, Phoma* sp, *Cladosporium* sp and *Geotrichum candidum* fungi from tomato during the period of investigation.

Ahmed *et al*. (2017) carried out a survey of fresh-market tomato fruit and to determine fungal and bacterial pathogens which were most commonly associated with postharvest diseases. They found that *Alternaria, Botrytis, Colletotrichum, Fusarium, Geotrichum, Mucor, Stemphyllium, Rhizopus* and *Penicillium* were the most frequently isolated fungi and *Acetobactor, Glucono bacter, Klebsiella, Leucontoc* and *Pectobacterim* were the prevalent bacteria.

Sajad *et al.* (2017) carried out an experiment with fungi which was associated with the spoilage of post harvest tomato fruit and he observed that tomato fruit had been suffered by fruit rot caused by *Alternaria alternata, Aspergillus niger, Geotrichum candidum, Alternaria solani, Mucor racemosus, Aspergillus flavus, Fusarium oxysporum, Fusarium moniliforme, Penicillium digitatum, Rhizopus stolonifer, Colletotrichum lycopersici, Sclerotium rolfsii, Myrothecium rodium, Phoma destructiva,* and *Trichothecium roseum.*

Samuel and Orji (2015) conducted an experiment to isolate, identify, and characterize the fungi associated with spoilage of tomato fruits in Nigeria and they found the fungi *Aspergillus niger, Rhizopus stolonifer, Fusarium oxysporum, Saccharomyces cerevisiae, Alternaria alternata, Penicillium digitatum* and *Geotrichum candidum.*

Lemma *et al*. (2014) carried out an experiment to identify the postharvest rotting microorganisms from tomato fruits in Ethiopia and they found *Erwinia carotovora, Clavibacter* sp, *Xanthomonas campestris, Ralstonia solanacearum, Pseudomonas aeruginosa*, *Alternaria* sp, *Fusarium* sp, *Penicillium* sp and *Rhizopus* sp as postharvest rotting microorganisms.

Ignjatov *et al.* (2012) conducted an experiment to isolate, determine, and identify causal organisms of tomato wilt and fruit rot and he found *Fusarium oxysporum* as causal agent.

Zhao *et al*. (2012) conducted an experiment about controlling of post harvest soft rot disease of tomato and they found *Erwinia carotovora* as a bacterial pathogen of soft rot disease of tomato.

Oladiran and Iwu (1993) stated that seven fungi associated with fruit rot of tomato were isolated including *Fusarium equiseti, F. chlamydosporum, Alternaria solani, Geotrichum candidum, Acremonium recifei, Aspergillus flavus* and *A. niger*.

2.2. Isolation and identification of postharvest diseases of carrot

Hussain (2018) carried out an extensive survey in Baghdad market. He isolated fungi associated with post-harvest carrot *(Daucus carota* L.) degradation. He found five fungal species, such as, *Aspergillus niger*, *Alternaria radicina*, *Sclerotinia sclerotiorum*, *Geotrichum candidum* and *Rhizoctonia carotae* from decayed sample.

Akhtari *et al*. (2016) conducted a survey on fungi associated with post-harvest deterioration of carrot and radish storage roots. They found *Aspergillus niger, Geotrichum candidum* and *Rhizopus oryzae* from the rotten samples.

Tahzima *et al.* (2014) described about the bacterial disease of carrot and they isolated the bacteria *Candidatus Liberibacter solanacearum.*

Zhang *et al.* (2014) worked on carrot in China. They found *Fusarium oxysporum* and *Fusarium solani* causing fusarium dry rot of carrot.

Godfrey and Marshall (2002) conducted an experiment to identify the bacteria from carrot causing bacterial soft rot disease during refrigeration in New Zealand and they found *Pseudomonas viridiflava* and *Pseudomonas marginalis* as bacterial pathogen from carrot.

El – tarabily *et al.* (1996) worked on cavity spot diseases of carrots in Western Australia and they isolated *Pythium sulcatum* fungi.

Khan *et al.* (1992) stated that *Escherichia coli, Klebsiella, Enterobacter,* and *Serratia* were lactose fermenters while *Pseudomonas, Shigella* and *Acinetobacter* were non-lactose fermenters and all bacteria were isolated from fresh samples of cucumber, carrot and lettuce.

2.3. Isolation and identification of postharvest diseases of cucumber

Mukhtar *et al.* (2019) worked with sweet oranges, cucumber and lettuce in Sharada market, Kano State-Nigeria and they isolated and identified the fungi namely- *Aspergillus fumigates, Aspergillus niger* and *Fusarium oxysporum* from cucumber. They also tested the pathogenicity tests in cucumber.

Nazerian *et al.* (2011) carried out an experiment about soft rot on cucumber in Malaysia and they isolated *Pectobacterium carotovorum* subsp *carotovorum* as a causal agent.

Majdah (2008) carried out an experiment with cucumber at Dere'iyah region, Riyadh district and he found 35 fungal isolates belonging to 4 species, namely, *Aspergillus niger, Fusarium oxysporum, F. solani* and *Rhizoctonia solani*. The four species were different in their prevalence as well as in their relative pathogenicity to cucumber both *in vitro* and *in vivo*.

2.4. Isolation and identification of postharvest diseases of capsicum

Frimpong *et al.* (2019) worked on pepper and isolated the fungi associated with deteriorated pepper. They found *Aspergillus* sp, *Fusarium* sp and *Colletotrichum* sp from pepper.

Akinyemi and Liamngee (2018) conducted an experiment to isolate and identify of fungi causing decay in pepper from selected markets in Makurdi and they isolated *Aspergillus niger, Fusarium moniliforme, Colletotrichum asianum, Fusarium oxysporum* and *Bipolaris zeicola* from pepper.

Lema *et al.* (2018) carried out an experiment to determine the fungal species affecting different pepper varieties in Dutsin-ma town, Katsina State and they found *Aspergillus flavus, Aspergillus fumigates, Aspergillus niger, Fusarium oxysporum, Lasidiplodia theobromae, Penicillium corylophilum, Rhizopus stolonifer* and *Verticillium* sp from *Capsicum annum.*

Fatimoh *et al.* (2017) worked on pepper and isolated the fungi associated with deteriorated pepper. They found *Rhizopus stolonifer, Aspergillus niger, Fusarium solani, Alternaria alternata, Geotrichum candidum, Mucor pussilus* from pepper fruit.

Samiah and Al-Mijalli (2014) conducted an experiment to isolate and characterization of plant and human pathogenic bacteria from green pepper (*Capsicum annum* L.) in Riyadh, Saudi Arabia and they found the bacteria namely, *Serratia* sp, *Ralstonia* sp, *Xanthomonas* sp. *Klebsiella* sp and *Proteus* sp.

Golkhandan *et al.* (2013) carried out an experiment about soft rot of pepper fruits in Malaysia and they found *Pectobacterium carotovorum* subsp *carotovorum* as a causal agent.

Ibrahim *et al.* (2002) carried out an experiment about bacterial spot disease of sweet pepper in Saudi Arabia and they isolated *Xanthomonas campestris* pv. *vesicatoria* as a causal agent.

2.5. Symptomatology of different postharvest diseases of tomato

Etebu *et al*. (2013) stated that sour rot caused by *Geotrichum candidum* is characterized by a lesion whose development resembles a cottage cheese like, dense, gelatinous mass. In early stages of diseases, the lesion is normally watery and later becomes covered with pathogen growth and stays relatively firm. They also stated about more postharvest diseases of tomato.

Etebu *et al.* (2013) also stated that buckeye rot caused by *Phytophthora parasitica* is characterized by water-soaked circular spots. As the diseases advance, the middle of the spots become blurred and overgrown with the fungus' sparse white mycelia.

Etebu *et al.* (2013) also reported about black mold diseases. Black mold rot is caused by several different fungal pathogens which include *Alternaria arborescens, Stemphyllium botryosum* and *S. consortiale*. They described that the diseases symptom is characterized by rots on the shoulder, stem, scar on tomato fruit blossom end. Lesion also occurred internally if it infects the stylar pore or a vascular strand connected to the stem scar.

Bacterial spot and bacterial canker diseases symptoms were described by Singh, 1998 and Mehrotra and ashok, 1980. They described that bacterial spot caused by several bacterial species of the genus *Xanthomonas*. Bacterial spot symptoms showed that spots on green fruit first appear as black, raised, pimple like dots surrounded by water soaked area. As the spots enlarge, with sunken, pitted centers, they are gray brown and scabby.

Bacterial canker is caused by the bacterium *Clavibacter michiganensis* sub sp. *michiganensis.* Infected fruit with white haloes of 16-32 mm are distinguished by dark colored necrotic lesions. The lesion appears white and slightly elevated at the beginning, but turns brown as the disease progresses and gradually becomes darkly colored.

Ignjatov *et al.* (2012) worked on tomato fruit and observed that *Fusarium oxysporum* as causal agent of tomato wilt and fruit rot. He stated that symptom of fusarium rot on fruits in the storage and warehouses appeared as white mycelium.

Wani (2011) reported that *Phytophthora* rot of tomato is caused by *Phytophthora infestans.* The symptoms of tomato phytopthora rot occurred as stiff, lumpy, reddish, brown lesion with indefinite margin on affected fruits, but soft consistency formed under wet conditions and he named this rot as dry phytophthora rot.

Wani (2011) also described about blue mold diseases. The symptom arose when small water soaked lesion further formed into watery rot at the wound site.

Hassan (1996) described about mucor rot diseases and noticed that a grayish green or brown with a water soaked spot that forms where the fruit touches the soil was the fiest indication on the tomato fruits. Lesions that widen on the surface of infected fruit have a characteristic pattern of alternating light and dark brown condensed rings that resemble the marking that formed as in buckey rot lesions that have a smooth surface and lack a margin that is clearly defined.

Sokhi and Sohi (1974) reported about the buckeye rot diseases. They described that the standard tomato lesions are brown to black, flattened or slightly sunken with an indefinite margin.

2.6. Symptomatology of different post harvest diseases of carrot

Chandrashekar *et al.* (2018) reported about bacterial soft rot of carrot caused by *Klebsiella variicola* in India. They described the symptoms associated with a

foul odour, wilting, collapse of foliage and water-soaked lesions developed on whole roots.

Stankovic *et al.* (2015) carried out an experiment about fusarium root rot of stored carrot and they described the symptoms included dry rot of the collar and crown as well as large, brown to dark brown, circular, sunken lesions on the stored roots. They also observed that abundant whitish mycelium covered the surface of the colonized roots.

Aktaruzzaman *et al.* (2014) observed that typical symptom of gray mold rot showed abundant blackish gray mycelia and conidia on infected root.

Tahzima *et al.* (2014) described about the bacterial disease of carrot caused by *Candidatus Liberibacter solanacearum.* They observed the symptoms of yellowing, purpling and curling of leaves, proliferation of shoots and formation of hairy secondary roots.

Farrar *et al.* (2004) reported that black rot of carrot is characterized by dry, black, sunken lesions on carrot roots.

Kora *et al.* (2003) carried out an experiment about sclerotinia rot of carrot. They described the symptoms of carrot rot that lesions on stored roots that are infected from the field develop in the crown region as localized softened tissue and white mycelial tufts erupting through the cuticle.

2.7. Symptomatology of different post harvest diseases of capsicum

Ramdial *et al.* (2016) carried out an experiment about detection and molecular characterization of benzimidazole resistance among *Colletotrichum truncatum* isolates infecting bell pepper in Trinidad. In this experiment they described the anthracnose of bell pepper symptoms. He stated that water soaked, oval or angular sunken lesions were fruit symptoms that might develop as a single large lesion or as several smaller lesions that covered most fruits.

Gillis *et al.* (2014) carried out an experiment about bacterial soft rot disease of bell pepper and they found that fruits were affected by water-soaked lesions.

Golkhandan *et al.* (2013) described about bacterial soft rot disease of bell pepper fruits (*Capsicum annum*). They stated that water-soaked lesions were observed initially. Then peduncle, calyx tissues and entire fruits were turned into watery masses within 2 to 6 days.

Ibrahim *et al.* (2012) stated about the symptoms of bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria* on sweet pepper. They described that the bacterial spot disease was characterized by blister-like, raised, rough lesions.

Colletotrichum capsici induced anthracnose of sweet bell pepper is characterized by diseases fruit with brown to black necrotic patch. Fruits develop water-soaked and small black circular spots on the surface. (Warin *et al*., 2009; Chanchaichaovivat *et al*., 2007; Roberts *et al*., 2001).

Harp *et al.* (2008) conducted an experiment about the etiology of recent pepper outbreaks in Florida and he described that anthracnose disease is characterized by sunken, necrotic lesions on the pepper fruit surface that typically contain an abundance of conidia colored with tan or salmon.

Lowis Ivey *et al.* (2004) worked on bell pepper to identify and manage of *Colletotrichum acutatum* from immature bell peppers and he stated that symptoms of the anthracnose fruit were circular or angular sunken lesions containing conidial masses of pink to orange. Lesions could coalesce under extreme pressure.

Riva *et al.* (2004) carried out an experiment about bacterial spot disease in *Capsicum annum* and they found typical necrotic symptoms on *capsicum annum* L.

2.8. Overview of modified post harvest packaging and different storage conditions of different salad vegetables

Tsegay *et al.* (2013) worked with two varities of sweet bell pepper (*Capsicum annum* L.) - (Telme-red and velez – yellow) and they determined the effects of 0, 25, 50, 75 and 100 % fruit colorations and storage duration 0, 1, 2, 3 and 4 weeks on physiological quality and shelf life of two varieties of sweet pepper. They found that telmo variety gave significantly better post harvest quality and storability than velez variety.

Ilić and Vukosavljević (2010) stated that refrigeration is one of the most frequently used cooling methods and cold chain (supply chain of fruits and vegetables with controlled temperature) also practiced for minimizing the losses throughout the entire storage and distribution system. Cold chain has also reduced losses across the entire storage and distribution system.

Nyanjage, *et al.* (2005) worked with sweet pepper (California wonder) which was tested under three different packages (open trays, non-perforated and perforated polythene bags) stored at temperatures of 4^0C, 6.5^0C and ambient 17^0C for 25 days and he collected data on weight loss, skin green colour retention, incides with chilling injury and diseases. They also found that perforated polythene and storage temperature 6.5^0C gave the best results.

Rodrigues and Han (2003) desdribed that because of new packaging method active packaging is very useful for preserving the perishable fruits and vegetables. Different types of indicators (colour indicators, oxygen indicators and CO_2 indicators etc.) may also be used to warn the condition of the packaged product, enabling quality control of hygiene.

Mohamed *et al.* (1996) stated that shelf life of the product increased with the help of modified atmospheric packaging and they noticed that qualitative packaging life of the fruits were 4 and 3 weeks for 10^0C and 15^0C, respectively,

which were shown better result than one week in the case of without modified atmospheric packaging.

Fishman *et al.* (1996) described that perforated films used for fruit and vegetables packaging and kept the product healthy on the basis of the quantity of escaped gasses through the perforated layer as well as the rate of perforated film movement of gasses.

Lownds *et al.* (1994) worked with nine pepper bell cultivars (*Capsicum annum)* and they determined the water-loss rates, flaccidity, color and disease development when the cultivars were stored at 8^0C, 14^0C, or 20^0C for 14 days.

Sharp *et al.* (1993) studied that in the case of perforated film packaging, the cool air has been supplied for providing a suitable temperature to the packed fruits and vegetables.

Chapter III

MATERIALS AND METHODS

3.1. Experimental site

The experiment was conducted in the MS Laboratory of Department of Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University, Sher-e-Bangla Nagar, Dhaka-1207, Bangladesh.

3.2. Experimental period

The experiment was conducted during the period from March 2019 to March 2020.

3.3. Collection of samples

Salad vegetables were collected from local markets of Dhaka city during the time of January-February, 2019. During each visit 5 kgs of each sample were collected by random sampling from each market place. Total 15 samples were tested randomly for each salad vegetables.

3.4. Preservation of samples

The collected samples were washed properly to remove the soils and dust from the sample. After that surface sterilized with 70% ethanol, dried and kept in a polybag and stored in refrigerator at $(5\text{-}6)^0$ C for further study.

3.5. Isolation of causal organism

3.5.1. Preparation of Potato Dextrose Agar (PDA) media

As described by Hussain *et al.* (2012), potato dextrose agar (PDA) medium was prepared. In a conical flask containing 1000 ml distilled water, 200 g peeled potato extract, 20 g dextrose and 20 g agar was taken and mixed properly for the preparation of 1 liter PDA medium. The opening was sealed with cotton plug. It was autoclaved for 20 minutes at 121^0C under 15 PSI pressure. To

prepare the 250 ml medium, 20 drops of 50% lactic acid was added for avoiding the contamination of bacteria.

3.5.2. Isolation of different fungi from infected samples

The infected portion was surface sterilized with 70% ethanol. The infected portion along with the healthy portion of the sample was cut into small pieces (0.5-1.0 cm) and surface sterilized with 70% ethanol for 2-3 minutes. Then, the pieces were washed thrice with sterile water and the pieces were placed on sterilized filter paper to remove excess water adhering to the pieces. By maintaining equal distance, three pieces were plated in acidified PDA plates and in moist chambers aseptically (plate 1). The plates were incubated for 7 days at $25+1^0$C. After incubation period with the help of sterile needle, the fungal mycelia that grew over PDA were taken and transferred on new PDA plates in three replications. Then the plates were incubated for 7 days for 12 hours alternating cycles of light and darkness at $25+1^0$C temperature. After incubation, pure culture of the fungus was identified. The pure culture of the pathogens was preserved in PDA slants at 5^0C in refrigerator as stock culture for future use.

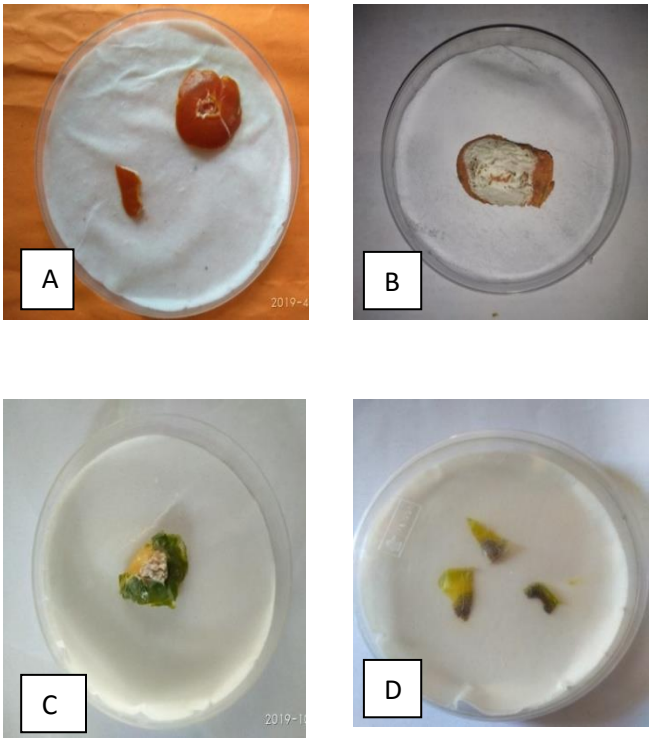

Plate 1. Different salad vegetable samples kept in moist medium

Figure A. Infected tomato in moist medium

Figure B. Infected carrot in moist Medium

Figure C. Infected cucumber in moist medium

Figure D. Infected capsicum in moist medium

3.6. Isolation and identification of bacteria from infected sample

3.6.1. Preparation of Nutrient Agar (NA)

Twenty eight gram dehydrated NA medium was taken in a conical flask containing 1000 ml distilled water. The medium was shaken thoroughly for few minutes to mix the ingredients properly. Then the media was autoclaved for 15 minutes at 121^0C under 15 PSI pressure.

3.6.2. Isolation of bacteria on NA media

The infected samples were rinsed properly with water. Then they were cut into small pieces. They were kept in 70% ethanol solution for 1 minute for surface sterilization of the infected samples. They were washed three times with distilled water. The cut pieces were kept in a test tube containing 6-7 ml of distilled water and macerated it with a sterile needle and kept for 30 minutes

for bacterial streaming. With the help of sterile pipette, one ml of this stock solution was transferred into a test tube containing 9 ml distilled water and shaken thoroughly resulting 1:10, 1:100, 1:1000, 1:10000 dilution. Similarly, final dilution was made up to 10^5. 0.1 ml of each dilution was spreaded over NA plate with the help of a sterile L shaped glass rod at three replications as described by Goszczynska and Serfontein (1998). After inoculated, the plates were incubated at 30^0C at inverted position. After 24 hrs incubation, the plates were observed and single colony was selected to restreak on new NA plate in three replications to get pure culture of the bacteria.

3.6.3. Preparation of Citrimide Agar

46.5 g Citrimide Agar was taken in 1000 ml water in an erlenmeyer flask. Then 10 ml glycerin was added in it. The mixture was boiled for mixing the elements properly. It was autoclaved for 20 minutes at 121^0C under 15 PSI pressure.

3.6.4. Growth of bacteria on Citrimide Agar

The pure colony growing on NA medium was transferred into citrimide agar medium by streak plate method. After inoculation, the plates were kept in an incubation chamber at 30^0C.

3.6.5. Growth of bacteria on Bacillus agar

Holbrook and Anderson developed Bacillus Cereus Agar, which is a highly specific and selective medium for the isolation (Holbrook *et al*., 1980). For *Bacillus* identification, 20.5 grams of Bacillus Cereus Agar was mixed in 475 ml distilled water. For dissolving it completely, the mixture was heated to boiling. The media was sterilized by autoclaving at 15Ibs pressure (121^0C) for 15 minutes. Then the medium was poured into sterile petridishes. Bacteria were restreaked for 24-72 hours. Examined daily for colony morphology and growth characteristics.

3.7. Biochemical test

For biochemical tests, single colonies of each isolate were used for biochemical tests. Many diagnostic and identification tests are based upon structural and chemical properties of bacteria (Lelliott and Stead, 1987).

3.7.1. KOH solubility test or Gram differentiation test

It is a quick method for gram differentiation of plant pathogenic bacteria without staining (Suslow *et al*., 1982). A single drop of 3% KOH (aqueous) was placed on a glass slide. One loop full of a single colony (18-24 hrs old) was taken from the NA plate using a cooled, sterile loop and it was mixed with KOH solution until an even suspension was obtained. The loop was raised a few centimeters from the glass slide and repeated strokes to have strands of viscid materials.

3.7.2. Gram's staining

A small drop of distilled water was mounted on a clean microscopic slide. Small portion of a young colony (24 hrs old) was removed from the nutrient agar medium with the help of a sterile loop, and then the bacterial smear was made on the slide. Bacterial film which spreaded thinly was air dried. Through the flame of a spirit lamp, underside of the glass slide was heated by passing it two times for fixing the bacteria on the slide. Then the slide was soaked with crystal violet solution for 1 minute. For a few seconds, it was rinsed under running tap water and excess water was removed by air. For a 1 minute, it was soaked with lugol's iodine solution. For 30 seconds, it was decolorized with 95% ethanol and again rinsed with running tap water and air dried. It was counterstained for 10 second with 0.5% safranine. It was rinsed under running tap water for a few seconds and excess water was removed by air. The glass slide was examined at 40x and 100x magnification using oil immersion.

3.7.3. Motility test

A semi-solid agar medium (SIM medium) was prepared in a test tube. Then a colony of young culture (18 to 24 hrs) was touched with sterile needle. Then a single stab down was made at the center of the tube to about half the depth of the medium. This was incubated at 35-37^{0}C and was examined daily upto 7 days to observe for a diffuse zone of growth flaring out from the line of inoculation. Diffuse, hazy growths that spread throughout the medium rendering it slightly opaque indicate positive result. The growth which is confined to stab line with sharply defined margins and leaving the surrounding medium clearly transparent indicate negative result.

3.7.4. Casein hydrolysis test

At first the skim milk agar plate was inoculated with the organism either a straight line or a zig-zag. The plate was incubated at 25^{0}C or 37^{0}C. The milk agar plate was observed for the presence or absence of a clear area or zone of proteolysis, surrounding the growth of each the bacterial test organism.

3.7.5. Citrate utilization test

18 to 24 hours old young culture was taken with sterile needle and was inoculated it in simmons citrate agar on slant in a test tube. The inoculum did not take from a broth culture because of heavy inoculum. This was incubated for up to 7 days at 35-37^{0}C. It was examined for development and growth of blue color, denoting alkalization.

3.7.6. Lactose fermentation test

With a sterile needle or loop, test microorganism was inoculated in the test tube with Phenol Red Lactose Broth. The tube was incubated for 18-24 hours at 35-37^{0}C. Longer incubation periods were required to confirm a negative result. A positive test consists of a color change from red to yellow, indicating a pH change to acidic. A negative test consists of a color change from magenta or hot pink in the presence of bases or alkali.

3.7.7. Sucrose fermentation test

With a sterile needle or loop, test microorganism was inoculated in the test tube with Phenol Red Sucrose Broth. The tube was incubated for 18-24 hours at 35-37^{0}C. Longer incubation periods were required to confirm a negative result. A positive test consists of a color change from red to yellow, indicating a pH change to acidic. A negative test consists of a color change from magenta or hot pink in the presence of bases or alkali.

3.7.8. Dextrose fermentation test

With a sterile needle or loop, test microorganism was inoculated in the test tube with Phenol Red Dextrose Broth. The tube was incubated for 18-24 hours at 35-37^{0}C. Longer incubation periods were required to confirm a negative result. A positive test consists of a color change from red to yellow, indicating a pH change to acidic. A negative test consists of a color change from magenta or hot pink in the presence of bases or alkali.

3.7.9. Oxidase test

1% aqeuos (w/v) solution of NNN'N-tetra-methyl-p-phenylene-diamine-dihydrochloride solution was spreaded on the middle of filter paper and the paper was placed on a petridish. Then some colony part of the bacteria were picked with sterile tooth pick and smeared onto the moistened filter paper and observed upto 60 seconds whether it changed color to dark purple or not.

3.7.10. Catalase test

A few drops of freshly prepared 3% H_2O_2 (Hydrogen peroxide) was added in a 48 hours old pure culture of bacterium grown on NA plate and examined whether it produced bubbles within a few seconds or not.

3.8. Pathogenicity test

Regarding pathogenicity test apparently healthy samples of uniform size were procured. These samples were washed with tap water to remove dust particles

dried. These samples were surface sterilized by dipping in 70% ethanol solution for one minute followed by three times washing in sterile distilled water to remove excesses 70% ethanol. The samples subjected to pathogenicity test were inoculated by following method:

For fungal isolates, a sterile five (5) mm cork borer was used to punch the salad vegetables and the discs removed. The same size of the cork borer was used to cut sections of each of the cultures of the previously isolated fungal pathogens and the discs were used to inoculate the healthy wounded salad vegetables. By using sterile transparent adhesive tape, the wound on the inoculated salad vegetables were sealed. The negative control was also set in the same manner but sterile PDA was used without fungal cultures. As a treatment, three salad vegetable samples were placed in each sterile polythene bag, replicated three times and stored at room temperature (25°C) in the laboratory.

For bacterial isolates, bacterial cell suspension (10^8cfu/ml) was prepared previously from 48 hrs old cultures and the surface sterilized selected samples were inoculated by puncturing the sample with the help of tooth pick containing bacterial cell suspension. In case of control, the tooth pick was dipped in sterile water then the sample was punctured with that and incubated at room temperature.

Observation data were recorded on the development of symptoms by constantly observing each and every inoculated sample daily. Any visual changes observed during incubation were recorded.

3.9. Genomic DNA extraction from Gram-negative bacteria

Protocol for DNA extraction of Gram negative bacteria (Promega):

1ml of over night culture was taken in a 1.5 ml micro centrifuge tube. This was centrifuged at 13000-16000 × g for 2 minutes to pellet the cells. The supernatant was removed. 600 µl of Nuclei Lysis Solution was added to this

and gently pipetted until the cells were resuspended. Then incubated it at 80^0C for 5 minutes to lyse the cells and cooled to room temperature. 3 µl of RNase solution was added to the cell lysate. Then the tube was inverted 2-5 times to mix. It was incubated at 37^0C for 15-60 minutes. The sample was cooled to room temperature. 200 µl of protein precipitation solution was added to the RNase –treated cell lysate. It was vortexed vigorously at high speed for 20 seconds to mix the protein precipitation solution with cell lysate. The sample was incubated on ice for 5 minutes. Then centrifuged at 13000-16000× g for 3 minutes. The supernatant containing the DNA was transferred to a clean 1.5 ml micro centrifuge tube containing 600 µl of room temperature isopropanol. It was mixed by inversion until the thread-strands of DNA formed a visible mass. It was centrifuged at 13000-16000× g for 2 minutes. Then carefully poured off the supernatant and drained the tube on clean absorbent paper. Then 600 µl of room temperature 70% ethanol was added and the tube was inverted several times to wash the DNA pellet. It was centrifuged at 13000-16000× g for 2 minutes and the ethanol was aspirated. The tube was drained on clean absorbent paper and the pellet was allowed to air dry for 10-15 minutes. 100 µl of DNA Rehydration solution was added to the tube and the DNA was rehydrated by incubating at 65^0C for 1 hour. The solution was mixed by gently tapping the tube. Alternatively, the DNA was rehydrated by incubating the solution overnight at room temperature or at 4^0C. Then the DNA was stored at 2-8^0C.

3.10. Determination of phylotype in PCR

Phylotype identification of each isolate was done by PCR. Phylotype specific PCR was carried out in 25 µl final volume of reaction mixture, containing 1x Hot Start Green Master Mix, (dNTPs, Buffer, $MgCl_2$, Taq Pol), Cat: M7432, Origin: Promega, USA, genomic DNA, distilled water and specific forward and reverse primers of targeting specific bands. The following cycling program was used in a thermal cycler: 95^0C for 3 mins, 95^0C for 30s, 48^0C for 30s, 72^0C for 90s, 72^0C for 5 mins followed by a final extension period of 4^0C overnight. A 5 µl aliquot of each amplified PCR product was subjected to electrophoresis on

2% agarose gel, Cat: V3125, Origin: Promega, USA. The PCR amplification was targeted 1465-bp.

3.11. Effect of different storage containers on postharvest deterioration of different salad vegetables

In different storage containers, salad vegetables are kept until they show different microbial growth on their surface. Each sample showed different symptoms on different storage containers. For each sample, minimum three samples were stored. Four different storage containers were used namely: polythene bag, net bag, plastic basket and clothing bag.

Chapter IV

RESULTS

4.1. Identification of postharvest diseases of salad vegetables by visual symptoms

4.1.1. For fungal pathogen

4.1.1.1. Visual symptoms of post harvest diseases of tomato

4.1.1.1.i. Sour rot disease of tomato

The fruit skin was ruptured and creamy white mycelia were found inside the tomato. The pulp was found very soft in infected areas (Plate 2, Fig. A).

4.1.1.1.ii. Aspergillus rot disease of tomato

Sunken lesion was found and blackish mycelia were observed over the infected area later on (Plate 2, Fig. B).

4.1.1.1.iii. Fusarium rot disease of tomato

The infected part of tomato was soft, curved and the skin of tomato became wrinkled and cottony soft whitish mycelia were observed (Plate 2, Fig. C).

4.1.1.1.iv. Green mold disease of tomato

The infected part became soft and the greenish spore around with whitish spore found in infected portion (Plate 2, Fig. D).

4.1.1.2. Visual symptoms of postharvest diseases of carrot

4.1.1.2.i. Fusarium rot disease of carrot

Whitish mycelium was found around the collar region of carrot and shrinkage was observed (Plate 2, Fig. E).

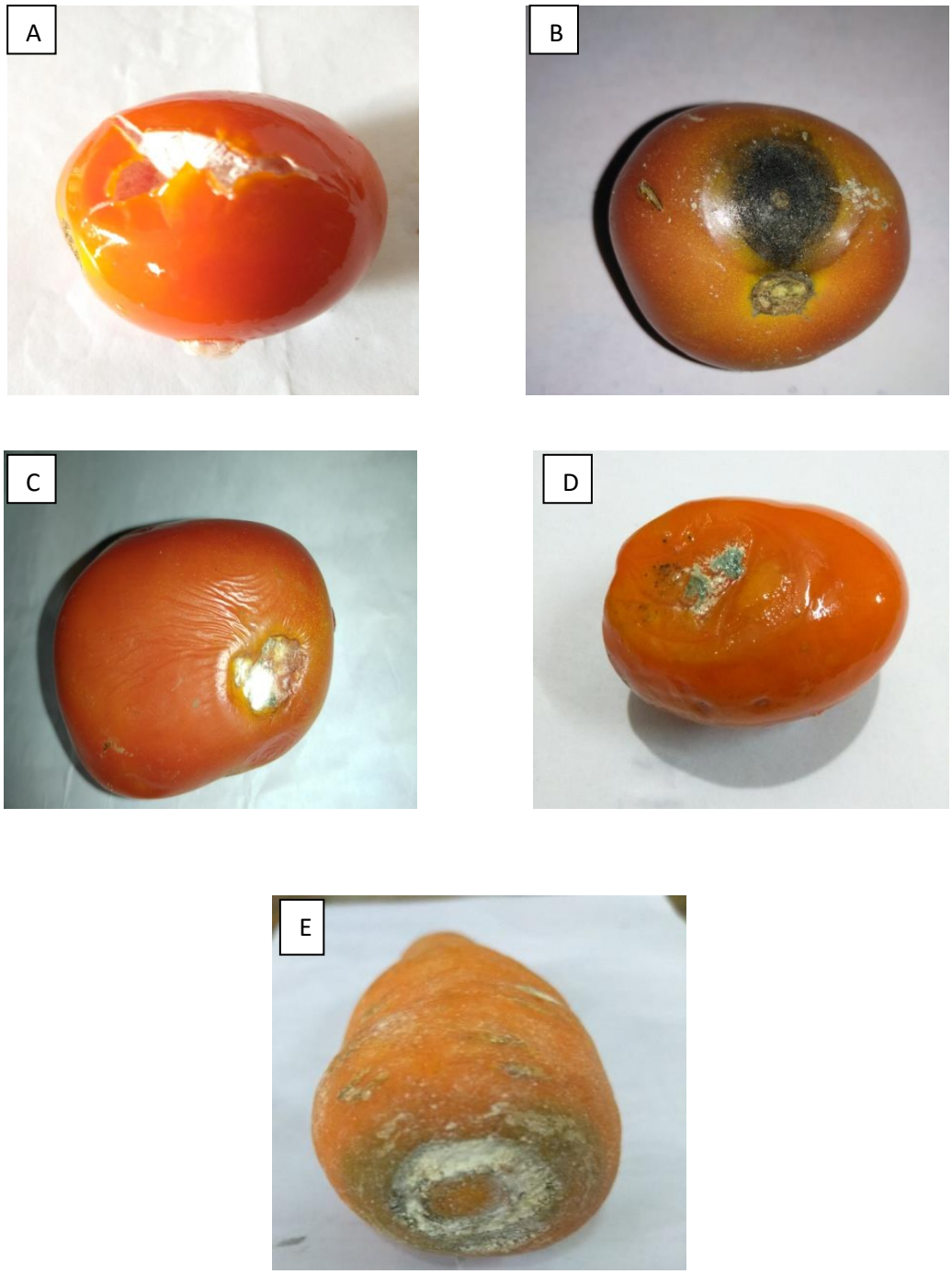

Plate 2. Visual symptoms of post harvest diseases of tomato and carrot

Figure A. Sour rot disease of tomato
Figure B. Aspergillus rot disease of Tomato
Figure C. Fusarium rot disease of tomato
Figure D. Green mold disease of Tomato

Figure E. Fusarium rot disease of carrot

4.1.1.2.ii. Aspergillus rot disease of carrot

The carrot became dried and shrinkage was observed. Blackish mycelium was found in the collar region of the carrot (Plate 3, Fig. A).

4.1.1.2.iii. Cottony soft rot disease of carrot

At first water soaked spots were found in the crown. Cottony, white fungal mycelium was also found in the collar region of the carrot (Plate 3 Fig. B).

4.1.1.3. Visual symptoms of postharvest diseases of cucumber

4.1.1.3.i. Fusarium rot disease of cucumber

Cottony, whitish mycelium was found around the cucumber. Shrinkage was also observed (Plate 3, Fig. C).

4.1.1.3.ii. Aspergillus rot disease of cucumber

Greenish white mycelium was found in the infected portion and infected portion became wetty and soft (Plate 3, Fig. D).

4.1.1.4. Visual symptoms of postharvest diseases of capsicum

4.1.1.4.i. Anthracnose disease of capsicum

Sunken lesions which produce conidial masses found the skin of capsicum (Plate 3, Fig. E).

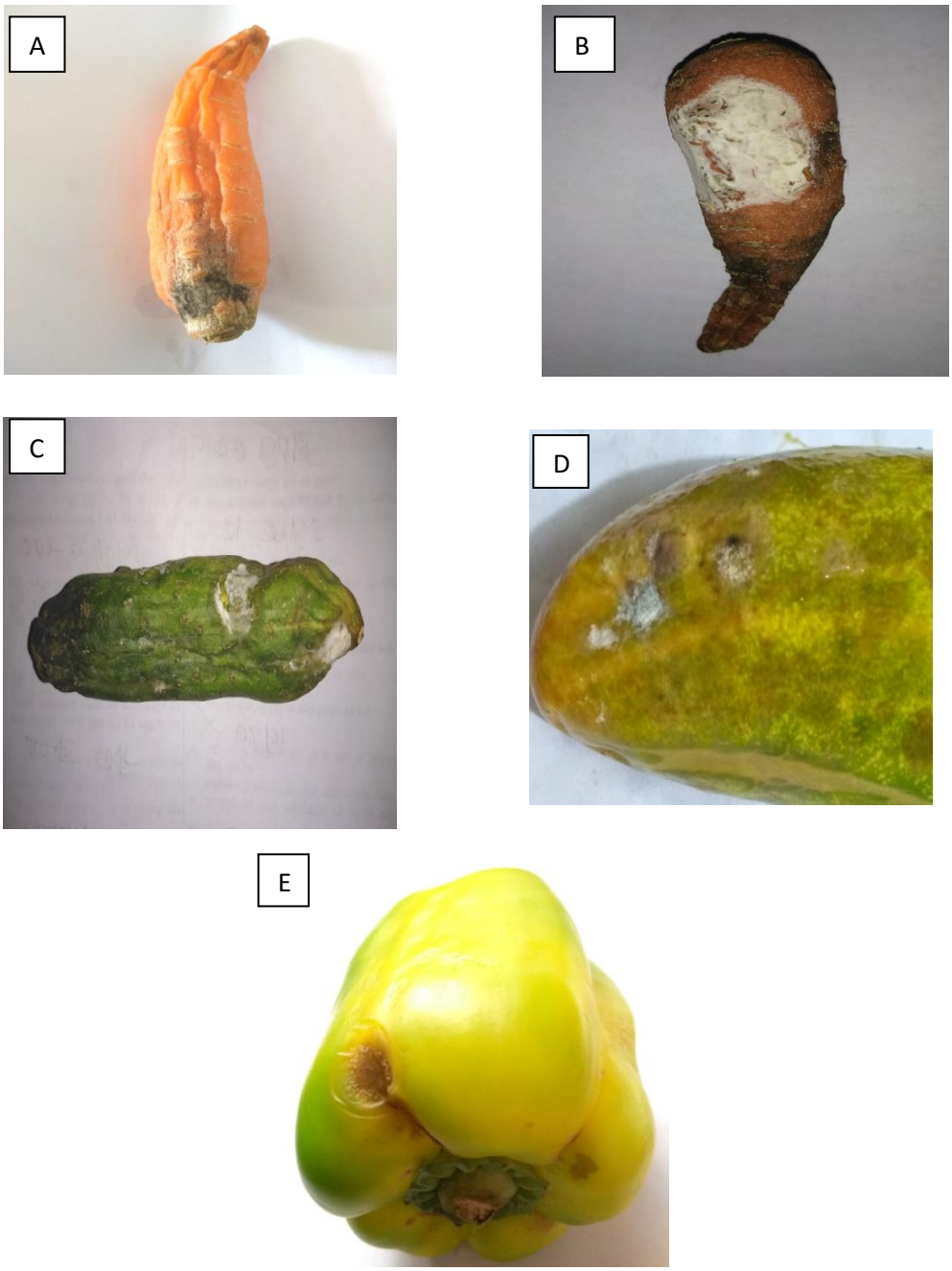

Plate 3. Visual symptoms of post harvest diseases of carrot, cucumber and capsicum

Figure A. Aspergillus rot disease of carrot

Figure B. Cottony soft rot disease of carrot

Figure C. Fusarium rot disease of cucumber

Figure D. Aspergillus rot disease of cucumber

Figure E. Anthracnose disease of Capsicum

4.1.2. For bacterial pathogen

4.1.2.1. Soft rot disease of tomato

Soft, watery sunken lesion was found on tomato (Plate 4, Fig. A).

4.1.2.2. Soft rot disease of cucumber

The infected cucumber was soft and water soaked lesion was found (Plate 4, Fig. B).

4.1.2.3. Soft rot disease of capsicum

The capsicum was soft and water soaked depressed lesions observed on it. Moreover bacterial ooze was emitted from infected capsicum (Plate 4, Fig. C).

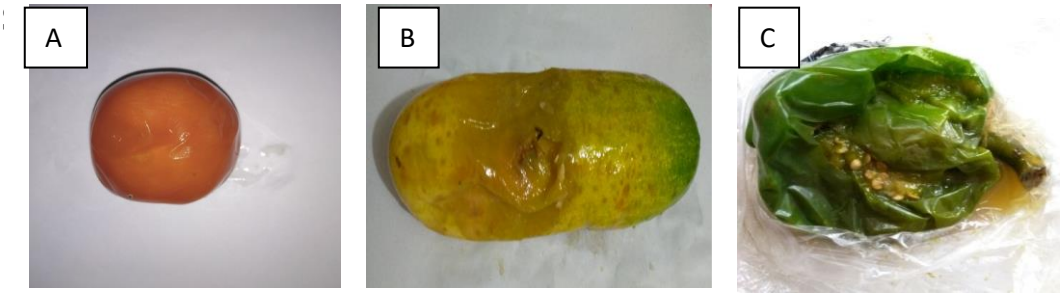

Plate 4. Visual symptoms of bacterial soft rot diseases of tomato, cucumber and capsicum

Figure A. Soft rot disease of tomato **Figure B. Soft rot disease of cucumber**

Figure C. Soft rot disease of capsicum

4.2. Isolation and identification of different fungi

4.2.1. Tomato

4.2.1.1. Cultural and microscopic characteristics of *Geotrichum candidum* (Sour rot disease of tomato)

The fungus colonies exhibit moderately rapid growth. The fungus produced off white to cream colored colonies with a butyrous texture with a velvety or matt appearance (Plate 5, Fig. A)

***Geotrichum candidum*:** The fungus produced hyaline (clear) septate hyphae which showed dichotomous branching. Advancing undifferentiated aerial hyphae produced chains of arthroconidia which fragmented into cells. The cells were cylindrical in shape (Plate 5, Fig. B).

4.2.1.2. Cultural and microscopic characteristics of *Aspergillus niger* (Aspergillus rot disease of tomato)

The fungus colonies on PDA exhibit whitish mycelial growth. At later stage, the colonies turned into blackish velvety appearance (Plate 5, Fig. C).

***Aspergillus niger*:** Colorless conidiophores were observed under the microscope. Conidial heads of the organism were globose and dark brown in color. Two series (biseriate) phialides were found covering the entire vesicle. (Plate 5, Fig. D).

4.2.1.3. Cultural and microscopic characteristics of *Fusarium* sp (Fusarium rot disease of tomato)

The fungus colonies on PDA exhibit fluffy, pinkish mycelium around with whitish mycelium (Plate 5, Fig. E).

***Fusarium* sp:** Few macro conidia and huge number of micro conidia were observed under the microscope (Plate 5, Fig. F).

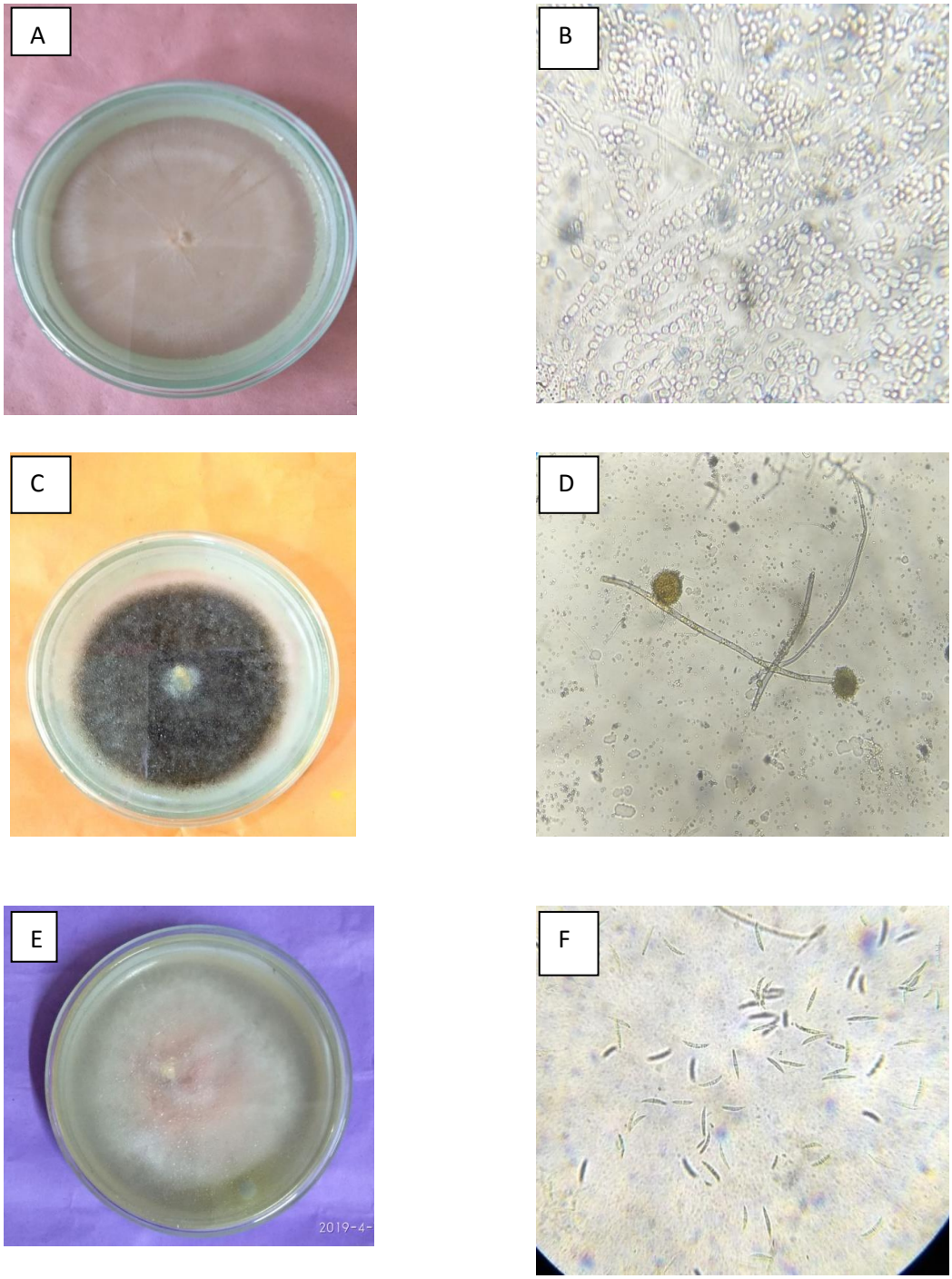

Plate 5. Cultural and microscopic view of isolated fungi from tomato

Figure A. ***Geotrichum candidum*** **on PDA**
Figure B. ***Geotrichum candidum*** **(40x)**
Figure C. ***Aspergillus niger*** **on PDA**
Figure D. ***Aspergillus niger*** **(10x)**
Figure E. ***Fusarium*** **sp on PDA**
Figure F. ***Fusarium oxysporum*** **(40x)**

4.2.1.4. Cultural and microscopic characteristics of *Trichoderma* sp (Green mold disease of tomato)

The fungus colonies exhibit rapid growth. The fungus produced the mycelia white with greenish yellow tint in color (Plate 6, Fig. A).

Trichoderma sp: The conidiophores observed to be long and less branches. The branches are relatively short and arising singly, alternately or pair from the main axis (Plate 6, Fig. B).

4.2.2. Carrot

4.2.2.1. Cultural and microscopic characteristics of *Fusarium* sp (Fusarium rot disease of carrot)

The fungus colonies on PDA exhibit fluffy, pinkish white mycelium (Plate 6, Fig. C).

Fusarium sp: Few macro conidia and huge number of micro conidia were observed under the microscope (Plate 6, Fig. D).

4.2.2.2. Cultural and microscopic characteristics of *Aspergillus niger* (Aspergillus rot disease of carrot)

The fungus colonies on PDA exhibit velvety, blackish mycelial growth (Plate 6, Fig. E).

Aspergillus niger: Colorless conidiophores were observed under the microscope. Conidial heads of the organism were globose, and dark brown in color (Plate 6, Fig. F)

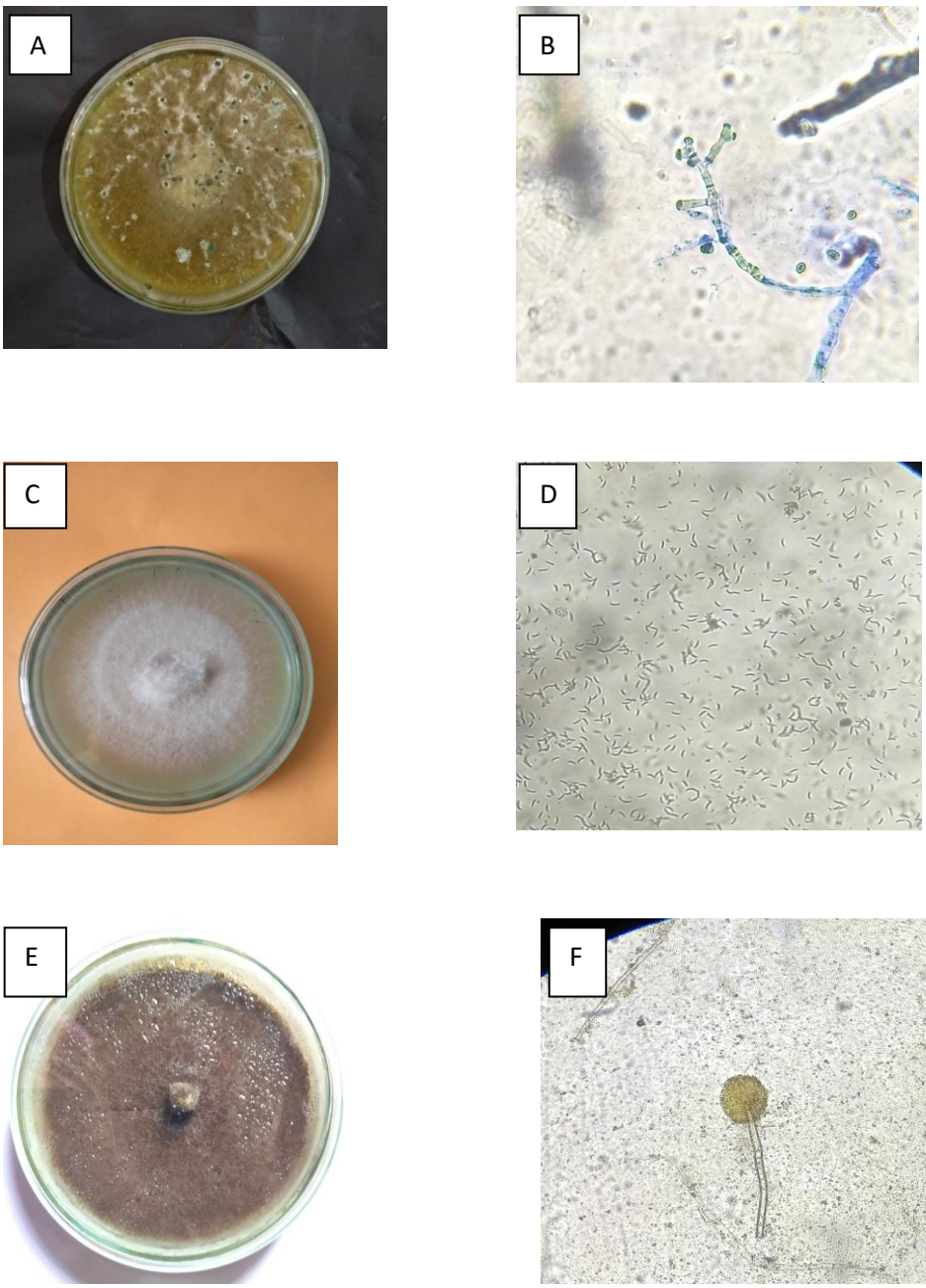

Plate 6. Cultural and microscopic view of isolated fungi from tomato and carrot

Figure A. *Trichoderma* sp on PDA
Figure B. *Trichoderma* sp (40x)
Figure C. *Fusarium* sp on PDA
Figure D. *Fusarium* sp (10x)
Figure E. *Aspergillus niger* on PDA
Figure F. *Aspergillus niger* (40x)

4.2.2.3. Cultural and microscopic characteristics of *Sclerotinia sclerotiorum* (Cottony soft rot disease of carrot)

The fungus colonies on PDA exhibit grayish mycelial growth and black sclerotia (Plate 7, Fig. A).

Sclerotinia sclerotiorum: The fungus produced hyaline and branched hyphae. Conidia or conidiophores were absent. (Plate 7, Fig. B).

4.2.3. Cucumber

4.2.3.1. Cultural and microscopic characteristics of *Fusarium* sp (Fusarium rot disease of cucumber)

The fungus colonies on PDA exhibit fluffy, pinkish, white mycelial growth (Plate 7, Fig. C).

Fusarium sp: The fungus produced few macro conidia and huge number of micro conidia (Plate 7, Fig. D).

4.2.3.2. Cultural and microscopic characteristics of *Aspergillus flavus* (Aspergillus rot disease of cucumber)

The fungus colonies on PDA exhibit fluffy, light greenish mycelial growth (Plate 7, Fig. E).

Aspergillus flavus: The fungus produced colorless conidiophores and globose conidial heads which were dark brown in color. (Plate 7, Fig. F).

4.2.4. Capsicum

4.2.4.1. Cultural and microscopic characteristics of *Colletotrichum capsici* (Anthracnose disease of capsicum)

The fungus colonies on PDA exhibit cottony and white to grayish mycelial growth (Plate 7, Fig. G).

Colletotrichum capsici: The fungus produced acervuli and setae (Plate 7, Fig. H).

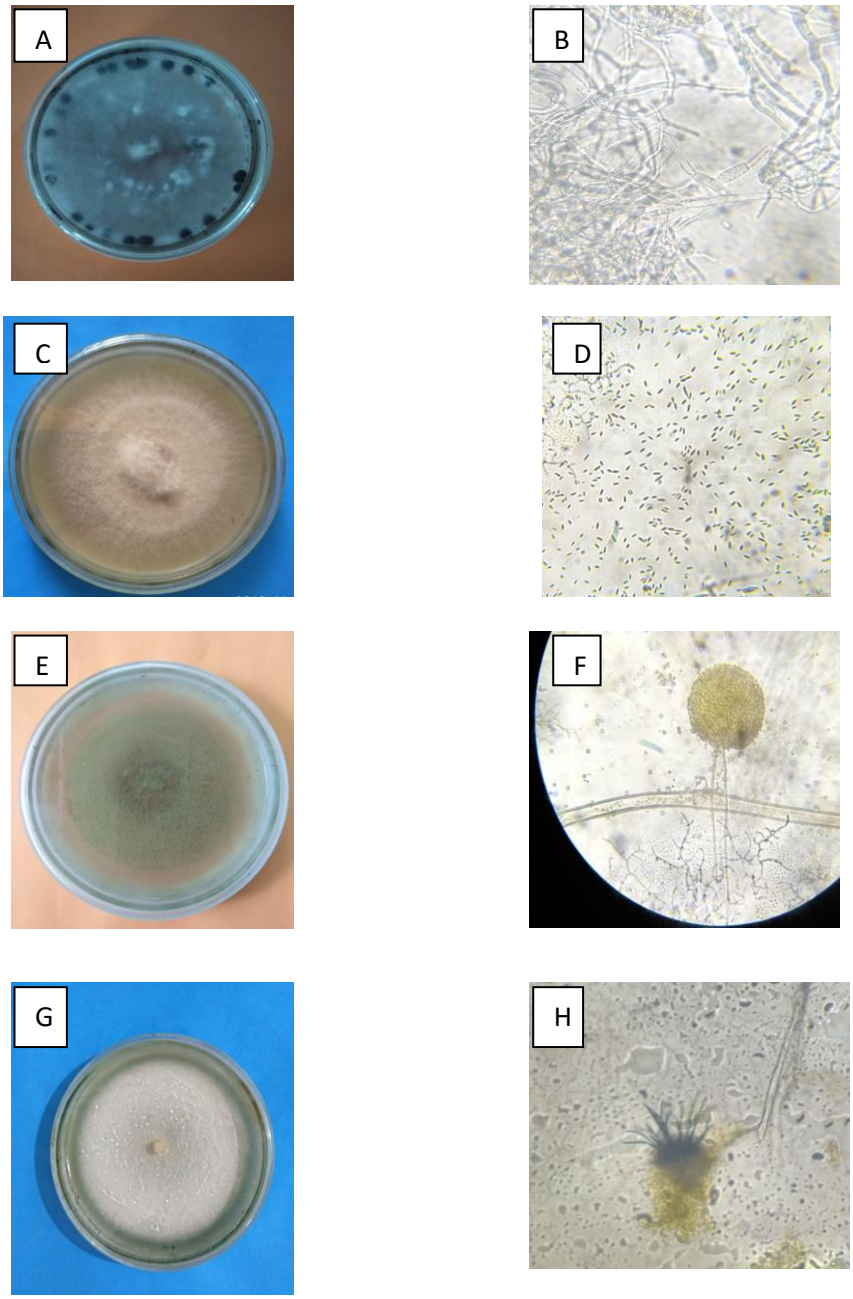

Plate 7. Cultural and microscopic view of isolated fungi from carrot, cucumber and capsicum

Figure A. *Sclerotinia sclerotiorum* on PDA
Figure B. *Sclerotinia sclerotiorum* (40x)
Figure C. *Fusarium* sp on PDA
Figure D. *Fusarium* sp (10x)
Figure E. *Aspergillus flavus* on PDA
Figure F. *Aspergillus flavus* (40x)
Figure G. *Colletotrichum capsici* on PDA
Figure H. *Colletotrichum capsici* (40x)

4.3. Isolation and identification of different bacteria

Several biochemical tests were conducted and some selective and semi-selective media were also used to identify and differentiate the bacteria.

4.4. Growth of different bacteria on NA plates

4.4.1. Soft rot disease of tomato: *Bacillus* sp (Plate 8, Fig. A and B).

4.4.2. Soft rot disease of cucumber: *Pseudomonas* sp (Plate 8, Fig. C and D).

4.4.3. Soft rot disease of cucumber: *Serratia marcescens* (Plate 8, Fig. E and F).

4.4.4. Soft rot disease of capsicum: The bacterium was unidentified (Plate 8, Fig. G and H).

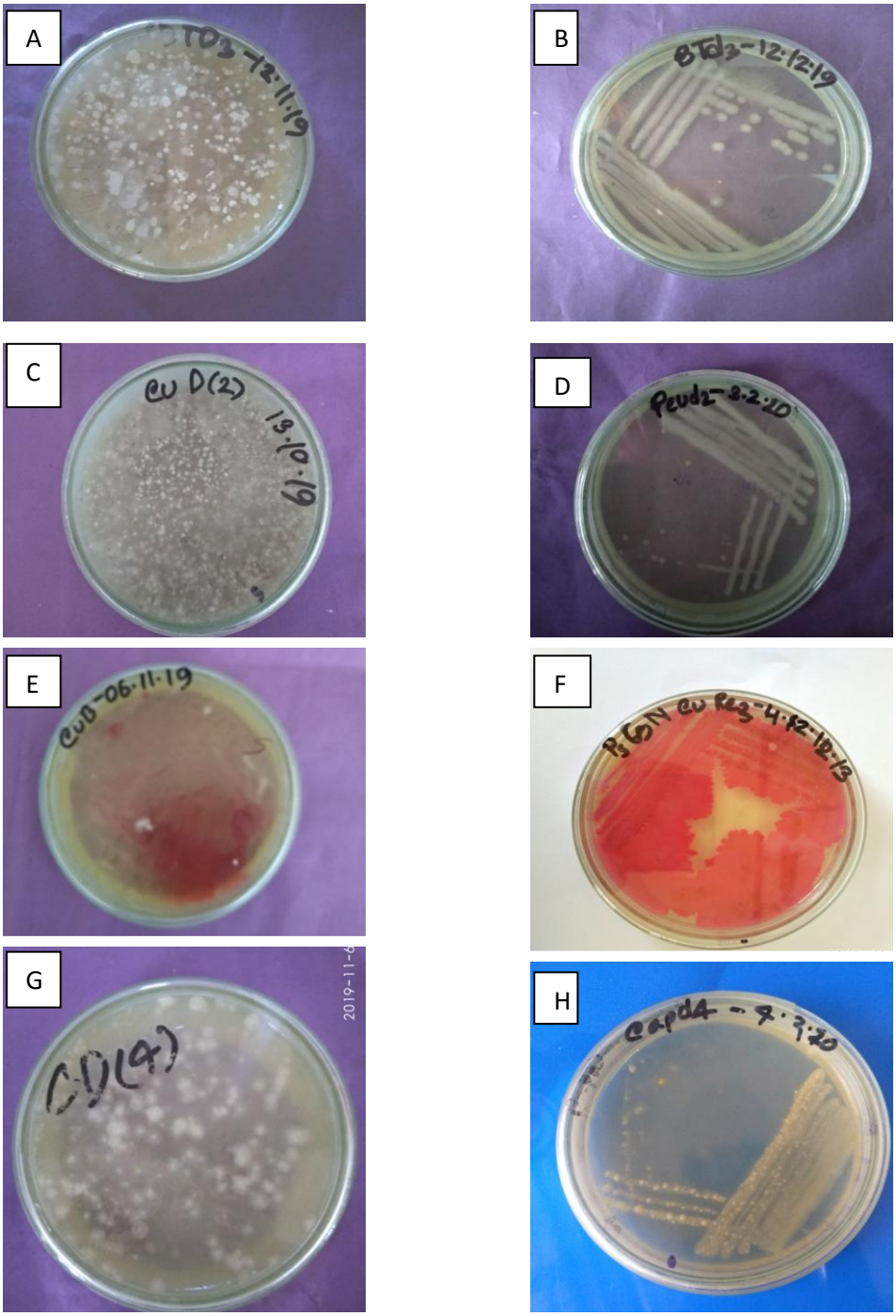

Plate 8. Bacterial growth on NA plates

Figure A. ***Bacillus*** **sp (spread plate method)**

Figure B. ***Bacillus*** **sp (streak plate method)**

Figure C. ***Pseudomonas*** **sp (spread plate method)**

Figure D. ***Pseudomonas*** **sp (streak plate method)**

Figure E. ***Serratia marcescens*** **(spread plate method)**

Figure F. ***Serratia marcescens*** **(streak plate method)**

Figure G. Unidentified bacteria (spread plate method)

Figure H. Unidentified bacteria (streak plate method)

4.5. Cultural characterization of different bacteria on NA plates and different biochemical test results

Cultural characteristics are tools of bacteria as identifying and classifying bacteria into taxonomic groups. When grown on a variety of media, bacteria exhibit differences in the microscopic appearance of their growth. These differences, called cultural characteristics are used as the basis for separating bacteria into taxonomic group. Cultural characteristics of different bacteria are shown in Table. 1 and different biochemical test results are shown in Table. 2.

Table 1. Cultural characterization of different bacteria on NA plates

Isolates	**Size**	**Form**	**Elevation**	**Margin**	**Pigmentation**	**Appearence**
Bacillus sp	Moderate	Circular	Raised	Undulate	Whitish	Shiny
Pseudomonas Sp	Small	Circular	Convex	Entire	Creamy	Shiny
Serratia marcescens	Large	Irregular	Raised	Entire	Reddish	Shiny
Unidentified	Small	Circular	Convex	Entire	Yellowish	Dull

Table 2: Biochemical tests for identification of different bacteria

Bacteria with sample	Motiliy Test	Casein test	Citrate test	Lactose test	Sucrose test	Dextrose test	Oxidase Test	Catalase test	Gram Reaction	KOH solubility test
Bacillus sp (Tomato)	+	-	+	Alkaline	Alkaline	Acid No gas	+	+	Short Rod Gram (+)ve	_
Serratia marcescens (Cucumber)	+	+	+	Acid/ Alkaline	Acid No gas	Acid Gas	_	+	Gram (-) ve	+
Unidentified (Capsicum)	_	_	+	Alkaline No gas	Acid Gas	Acid No gas	+	+	Gram (-) ve	+
Pseudomonas sp. *(*Cucumber)	+	+	+	Alkaline	Acid No gas	Acid No gas	+	+	Gram (-)ve Short Rod	+

4.6. Growth of different bacteria on citrimide agar medium

Figure 1. Isolation of *Pseudomonas* sp on citrimide agar medium

4.7. Growth of different bacteria on bacillus agar medium

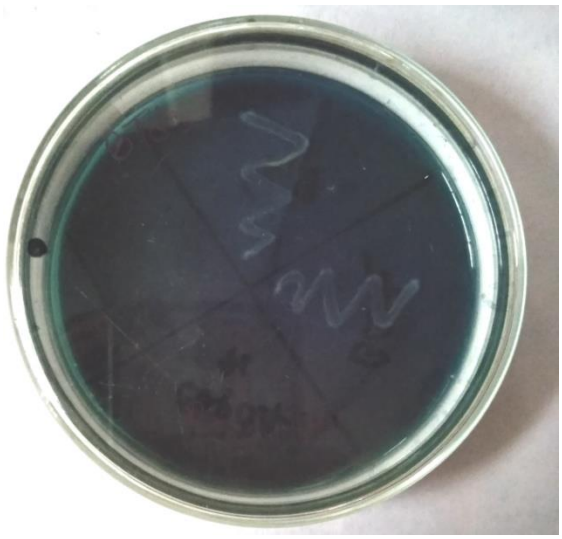

Figure 2. Isolation of *Bacillus* sp. on bacillus agar medium

4.8. Pathogenicity test

The study revealed that the micro-organisms isolated from the infected fruits were pathogenic but varied with pathogenicity. When inoculated into healthy fruit, pathogen caused the most rapid infection where the inoculated fruits were completely rotten by the end of the second day after inoculation. After inoculation by the pathogen, all fruits were showing their individual symptoms slowly. After two or three days, the fruits became completely rotten. From the rotten samples, pathogens were isolated and identified as described earlier to confirm pathogenicity (Plate 9).

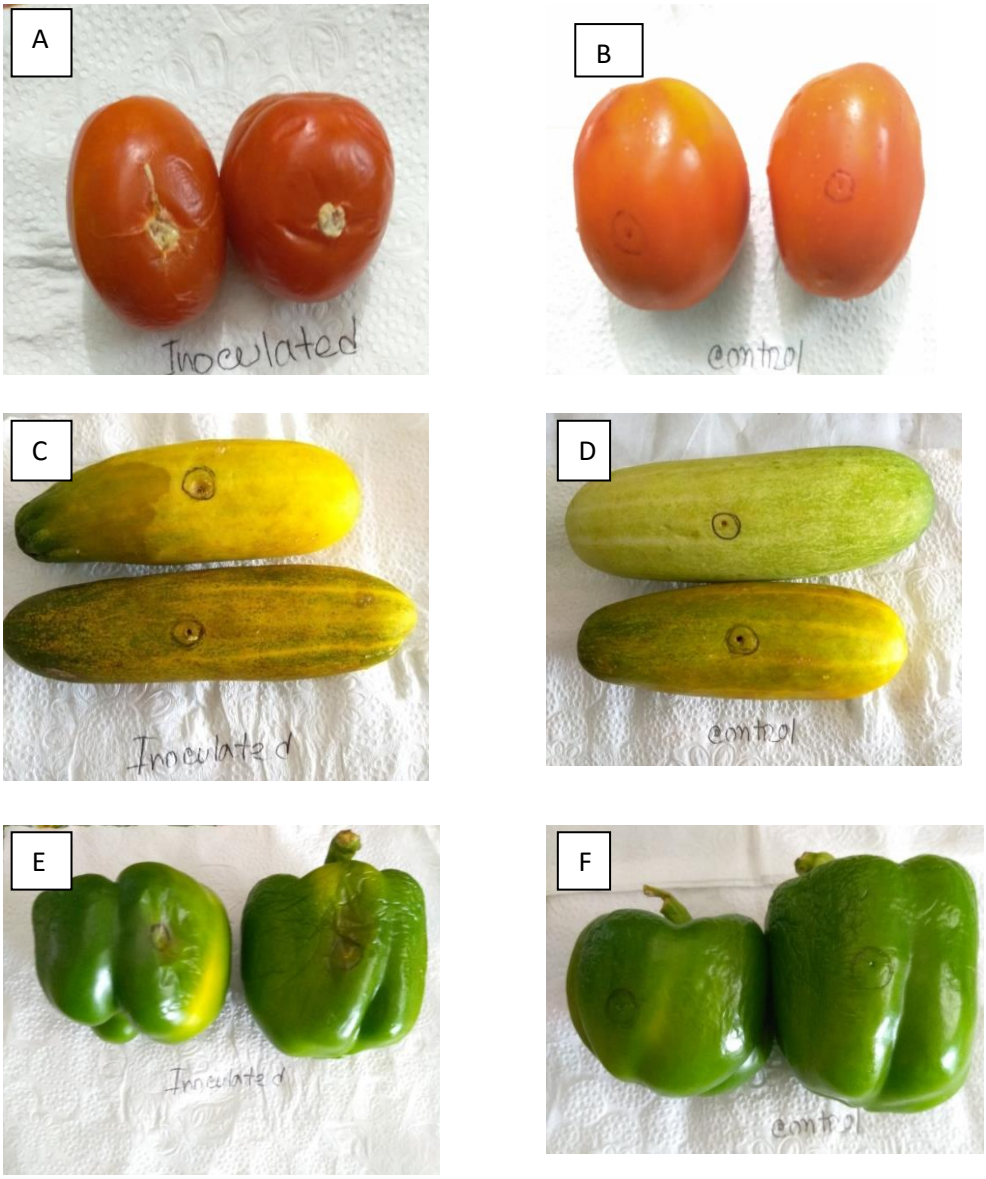

Plate 9. Pathogenicity test

Figure A. Inculated tomato with *Geotrichum candidum*

Figure B. Control (tomato)

Figure C. Inoculated cucumber with *Serratia marcescens*

Figure D. Control (cucumber)

Figure E. Inoculated capsicum with unidentified bacteria

Figure F. Control (capsicum)

4.9. DNA sequencing for Gram negative bacteria isolated from cucumber

4.9.1. Deposition of nucleotide sequences in GenBank of NCBI

DNA sequences of D1/D2 domain of 16S rDNA sequences of isolated bacteria were submitted to National Center for Biotechnology (NCBI) for deposition in the GenBank and obtain accession number. The obtained accession number MW404210 for *Serratia marcescens* strain SAU-1 (Fig. 3).

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1       AAACTCTACG ATGGGTGGAC TTCCTGAAGG CTAAGATTAC TACTAACTTT TGCAAACCAC
61      TCCCATGGTG TGACGGGCGG TGTGTACAAG GCCCGGGAAC GTATTCACCG TAGCATTCTG
121     ATCTACGATT ACTAGCGATT CCGACTTCAT GGAGTCGAGT TGCAGACTCC AATCCGGACT
181     ACGACGTACT TTATGAGGTC CGCTTGCTCT CGCGAGGTCG CTTCTCTTTG TATACGCCAT
241     TGTAGCACGT GTGTAGCCCT ACTCGTAAGG GCCATGATGA CTTGACGTCA TCCCCACCTT
301     CCTCCAGTTT ATCACTGGCA GTCTCCTTTG AGTTCCCGGC CGAACCGCTG GCAACAAAGG
361     ATAAGGGTTG CGCTCGTTGC GGGACTTAAC CCAACATTTC ACAACACGAG CTGACGACAG
421     CCATGCAGCA CCTGTCTCAG AGTTCCCGAA GGCACCAATC CATCTCTGGA AAGTTCTCTG
481     GATGTCAAGA GTAGGTAAGG TTCTTCGCGT TGCATCGAAT TAAACCACAT GCTCCACCGC
541     TTGTGCGGGC CCCCGTCAAT TCATTTGAGT TTTAACCTTG CGGCCGTACT CCCCAGGCGG
601     TCGATTTAAC GCGTTAGCTC CGGAAGCCAC GCCTCAAGGG CACAACCTCC AAATCGACAT
661     CGTTTACAGC GTGGACTACC AGGGTATCTA ATCCTGTTTG CTCCCCACGC TTTCGCACCT
721     GAGCGTCAGT CTTCGTCCAG GGGGCCGCCT TCGCCACCGG TATTCCTCCA GATCTCTACG
781     CATTTCACCG CTACACCTGG AAATTCTACC CCCCTCTACG AAACTTAGCT ATGCCAGTTT
841     CAAATGCAGG TTCCCGAGTT GAGGCTT
```

Figure 3. Nucleotide sequence of 16S rDNA from *Serratia marcescens* strain SAU-1 (Accession no. MW404210)

4.10. Effect of different storage containers on post harvest deterioration of salad vegetables

In different storage containers, salad vegetables were kept until they show different microbial growth on their surface. Each sample showed different symptoms on different storage containers. Effect of different storage containers are shown in Table 3.

Table 3: Effect of different storage containers keeping salad vegetables

Criteria	Tomato	Carrot	Cucumber	Capsicum
Effect of polybag	After 7 days, the tomatoes were found very soft, watery	After 10 days, the carrot became soft, watery	After 10 days, fungal attack were found among the cucumber	After 10 days, in case of all containers, the capsicums were found with black spot. (Plate 13)
	Plate 10, Fig. A	Plate 11, Fig. A	Plate12, Fig A	
Effect of clothing bag	After 10 days, fungal growth were found among the tomatoes	After 15 days, the carrots were covered with white mycelium	After 8 days, cucumber became yellowish and shrinked	
	Plate 10, Fig. B	Plate 11, Fig. B	Plate 12, Fig. B	
Effect of netbag	After 15 days, some tomatoes became shrinkage and some were attacked with whitish mycelium	After 25 days, the size of carrots were reduced	After 15 days, cucumber became dried and yellowish.	
	Plate 10, Fig. C	Plate 11, Fig. C	Plate 12, Fig. C	
Effect of plastic basket	After 20 days, one tomato was infected with black mycelium	After 12 days, the fungal growth was found in the carrot	After 10 days, all cucumber became yellowish	
	Plate 10, Fig. D	Plate 11, Fig. D	Plate 12, Fig. D	

4.10.1. Tomato

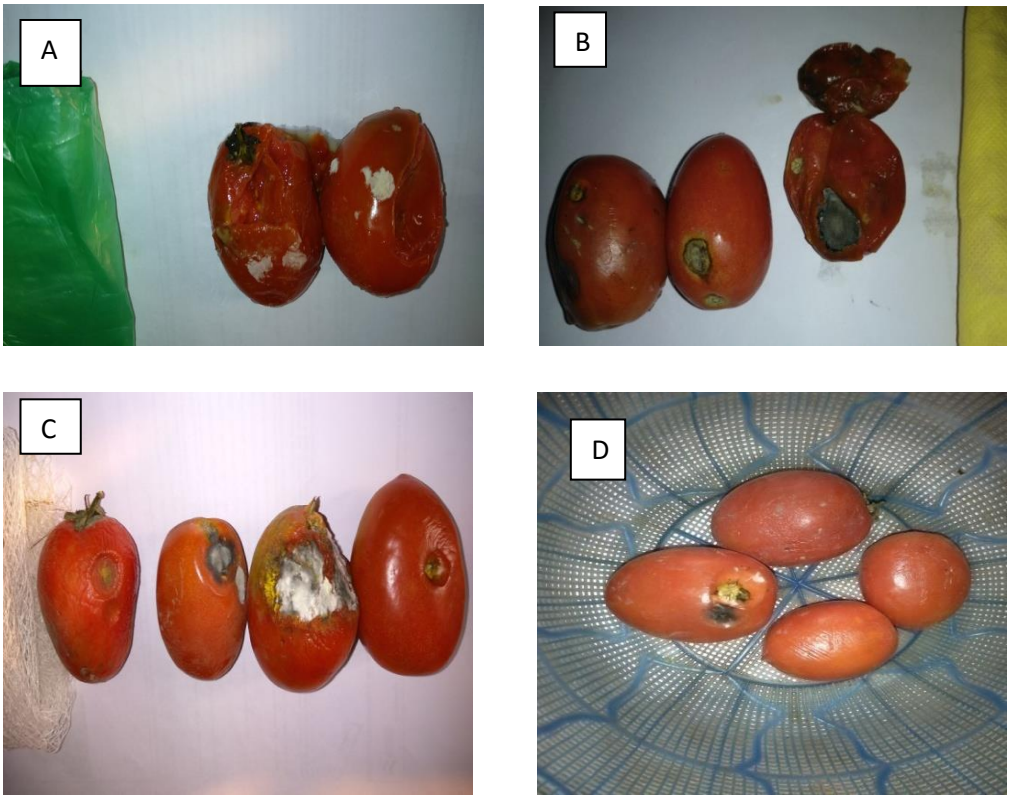

Plate 10. Symptoms of post harvest deterioration of tomato stored in different containers

Figure A .Effect of poly bag
Figure B. Effect of clothing bag
Figure C. Effect of net bag
Figure D. Effect of plastic basket

4.10.2. Carrot

Plate 11. Symptoms of post harvest deterioration of carrot stored in different containers

Figure A. Effect of poly bag

Figure B. Effect of clothing bag

Figure C. Effect of net bag

Figure D. Effect of plastic basket.

4.10.3. Cucumber

Plate 12. Symptoms of post harvest deterioration of cucumber stored in different containers

Figure A. Effect of polybag

Figure B. Effect of clothing bag

Figure C. Effect of net bag

Figure D. Effect of plastic basket

4.10.4. Capsicum

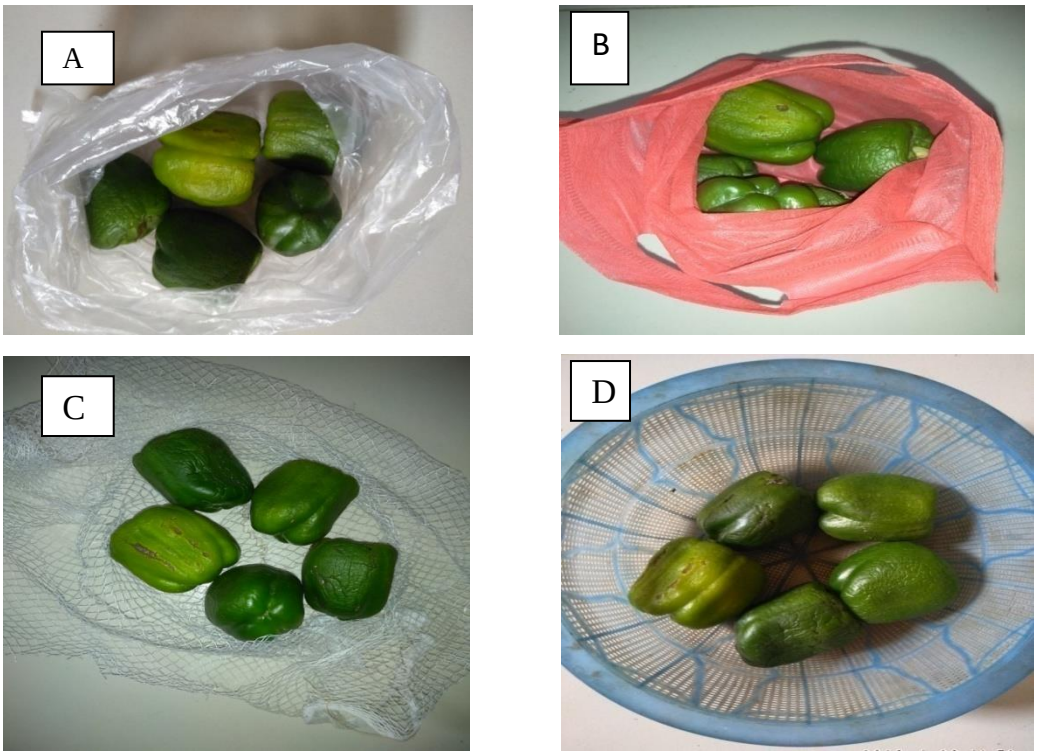

Plate 13. Symptoms of post harvest deterioration of capsicum stored in different containers

Figure A. Effect of polybag

Figure B. Effect of clothing bag

Figure C. Effect of netbag

Figure D. Effect of plastic basket

Chapter V

DISCUSSION

The laboratory experiment was conducted to identify the different post harvest diseases of selected salad vegetables and their causes. Pathogenicity tests of isolated organisms were carried out and molecular characterization of one bacterial isolate was carried out. Then effect of different containers on post harvest deterioration during storage was investigated. Four selected salad vegetables viz. tomato, carrot, cucumber and capsicum were used. Fungi and bacteria responsible for post harvest deterioration were isolated following tissue planting method and dilution method, respectively.

Losses caused by post-harvest diseases are greater than generally realized because the value of fresh fruits and vegetables increases several-fold while passing from the field to the consumer (Eckert and Sommer, 1967). At the market, the traders store the vegetables in bags and baskets and the fruits probably get contaminated during transportation and packaging in bags and baskets that are unsterilized.

The diseases were identified by visual observations and investigations. Aspergillus rot and fusarium rot both were observed on tomato, carrot and cucumber. Cottony soft rot of carrot, sour rot of tomato, green mold of tomato, anthracnose of capsicum and bacterial soft rot was developed on tomato, capsicum and cucumber.

Seven fungal species viz. *Aspergillus niger, Aspergillus flavus, Fusarium oxysporum, Trichoderma* sp, *Geotrichum candidum, Sclerotinia sclerotiorum, Colletotrichum capsici* and four bacterial strains namely *Bacillus* sp, *Pseudomonas* sp, *Serratia marcescens* and one unidentified bacteria were isolated from selected salad vegetables. Bacteria were separated by growing them on selective and semi selective media. *Bacillus* and *Pseudomonas* were separated by using bacillus agar medium by protocol described by Holbrook *et al.* (1980) and by using citrimide agar medium by following protocol described

by Brown and Lowbery (1965). Serial dilution method and spread plate method were followed to isolate the bacteria which are partially supported by Hasan and Zulkahar (2018).

The fungal pathogens associated with storage diseases of tomatoes were *Fusarium oxysporum, Aspergillus niger*, *Trichoderma* sp and *Geotrichum candidum* (Ogo-Oluwa and Kator, 2016; Samuel and Orji, 2015; Etebu *et al.*, 2013; Ignjatov, 2012).During storage various diseases destroy the tomatoes such as black mould rot (*Aspergillus niger*), Fusarium rot (*Fusarium* sp), gray mold rot (*Botrytis cinerea*), Aspergillus rot (*Aspergillus flavus*), blue mold (*Penicillium* sp), Phoma rot (*Phoma* sp), sour rot (*Geotrichum candidum*), Anthracnose (*Colletotrichum* sp) (Rodrigues and Kakde, 2019).The soft rot of tomato caused by *Bacillus* sp (Al-Allaf, 2020). Post harvest diseases caused by bacterial pathogens include the species of soft rotting genera *Erwinia, Clavibacter, Xanthomonas, Ralstonia, Pseudomonas* (Lemma *et al.,* 2014).

In this study *Bacillus* sp which was rod shaped showed positive results in Gram staining reaction, motility test, citrate utilization test, oxidase test, catalase test, and dextrose fermentation test and it showed negative results in lactose fermentation test, sucrose fermentation test, casein hydrolysis test and KOH solubility test which was partially supported by Rajsherkhar *et al.* (2017); Cappuccino and Sherman (2002).

The fungal pathogens associated with storage diseases of cucumber were *Fusarium* sp, *Aspergillus flavus, Aspergillus niger* (Mukhtar *et al.,* 2019). During storage various diseases destroy the cucumbers such as black mold (*Aspergillus niger*), Fusarium rot (*Fusarium* sp) (Majdah, 2008). Post harvest diseases caused by bacterial pathogen include the species of soft rotting genera *Pectobacterium, Pseudomonas* (Nazerian *et al.,* 2011). In this study, *Serratia* showed positive result in motility test, casein hydrolysis test, citrate utilization test, lactose fermentation test, sucrose fermentation test, dextrose fermentation test and catalase test, KOH solubility test. But it showed negative result in oxidase test and Gram staining reaction which was partially supported by

Abdullah *et al.* (2017). *Pseudomonas* sp. which was rod shaped showed positive result in motility test, casein hydrolysis test, catalase test, oxidase test, citrate utilization test, sucrose fermentation test, dextrose fermentation test, KOH solubility test and showed negative result in lactose fermentation test and Gram staining reaction. These findings were partially supported by Collins *et al.* (1996).

The fungal pathogen associated with storage diseases of carrot were *Fusarium oxysporum, Fusarium solani, Aspergillus niger, Sclerotinia sclerotiorum*. These findings were partially supported by a number of researchers (Madlao *et al.,* 2020; Hussain, 2018; Zhang *et al.,* 2014; Kora *et al.,* 2003). During the storage various diseases destroy the carrots such as sour rot (*Geotrichum candidum*), Aspergillus rot (*Aspergillus flavus, Aspergillus niger*), cavity spot (*Phythium sulcatum*), Fusarium rot (*Fusarium* sp) (Hussain, 2018; El-tarabily *et al.*, 1996). The post harvest diseases caused by bacterial pathogens include the species of soft rotting genera *Escherichia coli, Klebsiella, Enterobacter* (Khan *et al.,* 1992).

The fungal pathogens associated storage diseases of capsicum were *Aspergillus* sp, *Fusarium* sp and *Colletotrichum* sp (Frimpong *et al.,* 2019). During the storage various diseases destroy the capsicum such as Aspergillus rot (*Aspergillus niger*), Fusarium rot (*Fusarium* sp), anthracnose (*Colletotrichum capsici*), blue mold (*Penicillium* sp) (Lema *et al.,* 2018; Lewis *et al.,* 2004). Post harvest diseases caused by bacterial pathogens include species of soft rotting genera *Serratia, Klebsiella, Ralstonia, Xanthomonas* (Samiah and Al-Mijalli, 2014).The bacterium isolated from capsicum was unidentified. In biochemical test, it showed positive results in citrate utilization test, sucrose fermentation test, dextrose fermentation test, oxidase test, KOH solubility test and catalase test. And it showed negative result in motility test, casein hydrolysis test and lactose fermentation test.

Percentage of rotted salad vegetables affected by the materials that used in preparing the storage containers was differed according to the material used. Plastic basket was more suitable package used to store compared to others. On the other hand, containers made from polyethylene encouraged the bacterial soft rot of salad vegetables. This is due to increase RH around the salad vegetables associated with less ventilation.

DNA sequences of D1/D2 of 16S rDNA sequences of isolated bacteria from cucumber were submitted to National Center for Biotechnology Information (NCBI) for deposition in the GenBank and obtain accession number. The obtained accession number MW404210 for *Serratia marcescens* strain SAU-1.

In this study, seven fungal species, and four types of bacterial strains were identified. Further study is required to identify those pathogens and diseases caused by them.

Chapter VI

SUMMARY AND CONCLUSION

The present study was conducted to isolate and to identify the pathogen associated with infected salad vegetables. The experiment was conducted in the MS laboratory of Department of the Plant Pathology, Faculty of Agriculture, Sher-e-Bangla Agricultural University during the period from March, 2019 to March, 2020. The diseased samples were collected from local market places of Dhaka city. Seven fungal species and four bacterial strains were identified in this study. The fungal pathogen were isolated from infected samples and kept in the moist chamber. Then it transferred into PDA medium for their growth. The fungi namely *Aspergillus niger* (Aspergillus rot), *Aspergillus flavus* (Aspergillus rot), *Fusarium* sp (Fusarium rot), *Sclerotinia sclerotiorum (*cottony soft rot) *Geotrichum candidum* (Sour rot) and *Colletotrichum capsici* (Anthracnose) *Trichoderma* sp (Green mold) were identified.

In case of bacteria, *Serratia marcescens, Bacillus* sp, *Pseudomonas* sp (bacterial soft rot) were identified by visual observation of the symptoms. The bacteria were isolated following dilution plate method on NA medium. Out of four isolates, one was confirmed as *Bacillus* sp. by growing in Bacillus agar medium and one was confirmed as *Pseudomonas* sp by growing in citrimide agar medium where bacillus agar medium was a selective media for *Bacillus* sp and citrimide agar medium was a selective media for *Pseudomonas* sp. Bacterium isolated from capsicum was unidentified.

Pseudomonas sp. showed positive result in motility test, KOH solubility test, casein hydrolysis test, citrate utilization test, oxidase test and catalase test. *Basillus* sp gave positive result in motility test, citrate utilization test, oxidase test, catalase test and Gram reaction test. *Serratia marcescens* gave positive result in motility test, KOH solubility test, casein hydrolysis test, citrate utilization test, catalase test. The bacterium isolated from capsicum gave

positive result in citrate test, KOH solubility test, oxidase test, catalase test which was unidentified.

The cultural characteristics of the bacteria were also recorded. Pathogenicity test of some pathogen were also done.

DNA sequences of D1/D2 of 16S rDNA of *Serratia marcescens* isolate was submitted to National Center for Biotechnology Information (NCBI) for deposition in the GenBank and the accession number. The obtained accession number MW404210 for *Serratia marcescens* strain SAU-1.

The effects of different storage containers on different salad vegetables were also observed in this study. The study found that plastic basket was more suitable than the others to keep the samples in storage.

This research study was conducted based on the pathogen responsible for diseases salad vegetables. Further studies will be needed to determine the factors that influence the post harvest diseases of salad vegetables in the market place.

Chapter VII

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APPENDICES

Preparation of the culture media:

The composition of the media which is used in this thesis work are given below:

At 121^0C for 20 minutes all media are autoclaved at 15 lb pressure.

Potato Dextrose Agar (PDA)

Peeled potato extracts	200 g
Dextrose	20 g
Agar	20 g
Distilled Water	1000 ml

Nutrient Agar (NA)

Beef extract	3 g
Peptone	5 g
Bacto Agar	15 g
Distilled Water	1000 ml

Citrimide Agar

Pancreatic Digest of Gelatin	20 g
Potassium Sulfate	10 g
Magnesium Chloride	1.4 g
Cetyltrimethyl ammonium Bromide	0.3 g
Glycerine	10 ml
Agar	13.6 g
Distilled Water	1000 ml

Bacillus Agar

Peptone	1 g
Mannitol	10 g

Sodium chloride	2 g
Magnesium sulphate	0.1 g
Disodium phosphate	2.5 g
Monopotassium phosphate	0.25 ml
Sodium pyruvate	10 g
Bromothymol blue	0.12 g
Agar	15 g
Distilled Water	1000 ml

Sulfide Indole Motility (SIM) Medium

Pancreatic digest of casein	20 g
Peptic digest of animal tissue	6.1 g
Agar	3.5 g
$Fe(NH_4)_2(SO_4)_2.6\ H_2O$	0.2 g
$Na_2S_2O_3.5H_2O$	0.2 g
Distilled water	1000 ml

Skim Milk Agar

Skim milk powder	28 g
Tryptone	5 g
Yeast extract	2.5 g
Dextrose (glucose)	1 g
Agar	15 g
Distilled Water	1000 ml

Simmon Citrate Agar Medium

$NH_4H_2PO_4$	1 g
K_2HPO_4	1 g
NaCl	5 g
Sodium citrate	2 g
$MgSO_4$	0.2 g
Agar	15 g
Bromothymol blue	0.08 g
Distilled water	1000 ml

Phenol Red Carbohydrate broth

Tryticase or Protease Peptone No.3	10 g
Sodium Chloride (NaCl)	5 g
Beef extract (optional)	1 g
Phenol red solution	0.018
Carbohydrate source	10 g

Catalase reagent

From the H_2O_2 absolute solution, 3% aqueous solution of H_2O_2 was prepared.

Oxidase reagent

1% aqueous solution of NNN'N-tetramethyl-p-phenylene-diamine dihydrochloride was prepared from the absolute solution.

Gram staining reagents

Gram's Crystal violet (Hucker's modification)

Solution A: Crystal violet (90% dye content)	2 g
Ethyl alcohol	20 ml
Solution B: Ammonium oxalate	0.8 g
Distilled Water	80 ml

Solution A and B in equal volume to prepare crytal violet solution

Gram's Iodine (Gram's modification of Lugol's solution)

Iodine	1 g
Potassium iodiod (KI)	2 g
Distilled Water	300 ml

To prepare Gram's Iodine solution, add iodine after KI is dissolved in water.

Gram's alcohol (decolorizing agent)

Ethyl alcohol (95%)	98 ml
Acetone	2 ml
Safranin (2.5% solution in 95% ethanol)	10 ml
Distilled Water	100 ml

KOH solubility reagent

3% aqueous solution of KOH was prepared from the KOH granules

DNA Isolation:

1. Automated DNA extractor, Model: Maxwell 16, Origin: Promega, USA.
2. Homogenizer, Pro Scientific,

DNA Quantification:

1. NanoDrop Spectrophotometer, Model: ND2000, Origin: Thermo Scientific, USA.

PCR:

1. Gene Atlas, Model : G2, Origin: Astec, Japan.

Gel Electrophoresis System:

1. Horizontal, Model: Mini, Origin: CBS Scientific, USA.

Gel Documentation:

1. Alpha Imager, Model: mini, Origin: Protein Simple, USA.

PCR Clean-Up:

1. Centrifuge, Model: Kitman24, Origin: Tomy-Japan

DNA Isolation:

1. Maxwell Blood DNA Kit, Model: AS1010, Origin: Promega, USA.

PCR:

1. Hot Start Green Master Mix, (dNTPs, Buffer, $MgCl_2$, Taq Pol), Cat: M7432, Origin: Promega, USA.
2. Primer 27F: AGA GTT TGA TCM TGG CTC AG
3. Primer 1492 R: CGG TTA CCT TGT TAC GAC TT

Gel:

1. Agarose, Cat: V3125, Origin: Promega, USA.
2. 100 bp DNA Ladder, Cat: G2101, Origin: Promega, USA.
3. 1kb DNA Ladder, Cat: G5711, Origin: Promega, USA.
4. Diamond™ Nucleic Acid Dye, Cat: H1181, Origin: Promega, USA. (A Non Carcinogenic DNA Binding Dye).
5. TAE Buffer: Cat: V4251, Origin: Promega, USA.

PCR Clean Up:

1. SV Gel and PCR Clean Up System, Cat: A9281, Origin: Promega, USA.