ADDIS ABABA UNIVERSITY

SCHOOL OF GRADUATE STUDIES

FACULTY OF NATURAL SCIENCE

DEPARTMENT OF MICROBIA, CELLULAR AND MOLECULAR BIOLOGY

POST HARVEST BIOLOGICAL CONTROL OF BLUE MOLD ON ORANGE FRUIT

BY

LEMLEM MEQUANINT TENSAY

A Thesis Submitted to the School of Graduate Studies of Addis Ababa University in Partial Fulfillment of Degree of Master of Science in Biology (Applied Microbiology).

Jun, 2014
Addis Ababa

Table of Contents

Contents page

Table of content ……..I

List of tables……..IV

List of Figures……V

Acknowledgements …… ….VI

Abstract ……...……VII

Key termes ……..……VII

1. Introduction ……1

2. Objectives of the study……3

2.1Genereal objective……3

2.2. specific objectives……3

3. Literatur review……..……4

3.1.orange……4

3.2. orange production and utilization……..……..…….....4

3.3. Nutritinal value of orange ……..…….....7

3.4. Orange production in Ethiopia……..……..…..8

3.5. Importance of post harvest losses……..……9

3.6. Postharvest disease of orange fruit……12

3.6.1. Orange blue mold ……12

3.6.2. Causal organism……...12

3.6.3. The infection process……..……..12

3.6. 4. Symptoms……...…13

3.6.5.Control of blue mold after harvest…….....13

3.7. Post harvest biological control of fruit diseases…….....15

3.7.1. Opportunities for biological control of post harvest plant disease……...…17

3.7.2. Application of biocontrol agents against post harvest diseases ……..……18

4. Materials and methods…….……..19

4.1 Isolation of the pathogen……..…..…..19

4.2.Isolation of the antagonists……..……19

4.3. Characterization and identification of the pathogen……...……20

4.3.1 Morphological characterization of the pathogen……..…20

4.3.1.1 Macroscopic characterization of the pathogen…….…20

4.3.1.2 Microscopic characterization of the pathogen ……..……21

4.3.1.3. Biochemical characterization of the pathogen…….……..……21

4.4.Screening for antagonistic activity……...…22

4.4.1 Testing the potential of yeast antagonists…….……....22

4.5. In vivo test of antagonistic activity……23

4.5.1. Evaluation of antagonistic activity on artificially inoculated fruits……....23

4.5.2. Assessment of lesion …….....23

4.6. Characterization and identification of Antagonists…….....24

4.6.1 Morphological characterization…….....24

4.6.1.1 Growth on solid medium……...24

4.6.1.2 Growth in liquid medium……..……..25

4.6.2 Physiological characterization……...…25

4.6.2.1 Ascospore formation……..…..25

4.6.2.2 Fermentation of carbohydrates……..…..…26

4.6.2.3.Effect of Temperature on the growth of antagonists……..…26

4.7 Statistical Analysis……...……26

5. Result and discussion……..……27

5.1.Characterization and identification of the pathogen……..……27

5.1.1 Morphological and physiological characteristics of pathogen ……..27

5.1.2 Identification of orange blue mold causing organism……..……34

5.2.Characterization and identification of potential antagonistic isolates……...34

5.2.1 Morphological characteristics of potential antagonists……34

5.2.2 Biochemical haracteristics of potential antagonists……..……37

5.3 Inhibition of orange blue mold in vitro……..……38

5.4. In vivo Effect of Antagonists against orange blue mold Development……..…42

6. Conclusion……....44

7.Recommendation……...……44

8.Reference……..……..45

List of table

Table- 1: the growth morphology of orange blue mold causing organism on different media for 7-days……28

Tabel -2 the growth rate of orange blue mold incubated at different temprature on PDA for10 days……..30

Table-3: Microscopic and Macroscopic growth characteristics of potential yeast isolates on malt extract agar for 72h culture……35

Tabel-4 Physiological properties of potential antagonistic yeast isolate……...37

Table-5 the growth of potential antagonistic yeast isolates at different temperature on malt extract agar for 3 days……38

Table-6 inhibitory effect of yeast isolates against in vitro growth of orange blue mold……...…40

Table-7: Effect of antagonists on blue mold disease severity of artificially inoculated orange fruits……..……43

List of Figures

Fig.1: Colony growth characteristics of orange blue mold incubated at 25^0c on different media……..…29

Figure.2: the growth rate of orange blue mold causing organism on PDA at different temperature for 10 days……...……...…31

Fig-3:Form of penecilli of orange blue mold ……32

Fig-4: Symptoms of orange blue mold disease after inoculating the pathogen on artificiallywoundedorangefruit……33

Fig-5: colony and cell morphology of potential antagonistic yeasts grown on malt extractagarfor3days……36

Fig-7 Inhibitory effect of potential yeast isolates against orange blue mold on artificially inoculated orange fruits incubated for 12days at 25^0c…….……43

ACKNOWLEDGEMENTS

It gives me a great pleasure to acknowledge my advisor Dr. Dawit Abate for his in valuable guidance, advice, encouragement, support, and critical comments during the course of my study.

Together with this my sincere appreciation goes to Miss Zenebech and Miss Nigat, for their encouragement and cooperation in laboratory works.

I would like to thank Ato Taddess Abebayehu director of bole community school for his encouragement and cooperation in giving me time space to attend this program.

I would like to express my gratitude towards Ato Yonas Kefyalew for his encouragement and cooperation in materials.

I also enjoyed a lot to work with all my colleagues, mulatu, Solomon, aderaw, betearia and others. I appreciate all your support.

I am grateful to thank my family for their financial aid and encouragement through out this study. I do love you a lot my family.

ABSTRACT

Orange *(Citrus sinensis)* is among the most important fruit crop of Ethiopia. Blue mold is the most aggressive economically important wound parasite post harvest disease of orange fruit. The present study was under taken to isolate, characterize and identify indigenous biocontrol microorganisms (yeasts) occur on phyllospher of orange, mango, apple, lemon and avocado plants for their antagonistic activity against orange blue mold development as well as characterization and identification of orange blue mold causing organism has been done. In this study a total of 58 yeast isolates were isolated from fruit surfaces and leaves of orange, lemon, apple, mango and avocado plant. All of them were screened to test their antagonistic effect against orange blue mold development. Among the 58 isolates 9 isolates showed antagonistic activity against the development of orange blue mold. Three isolates namely isolate $\mathbf{Y_3}$, $\mathbf{Y_{10}}$ and $\mathbf{Y_{16}}$ were particularly effective in this respect with the antagonistic level of 32%, 36% and 38.7% respectively. All of them achieved significant disease suppression ($P<0.005$). The identity of the potential antagonists and the pathogen had been studded based on their morphological, biochemical and physiological characteristics. Isolate $\mathbf{Y_{10}}$ and $\mathbf{Y_{16}}$ were identified to the genus Rhodotorula and $\mathbf{Y_3}$ was belongs to Trichosporonoides and the pathogen was identified as *Penicillium italicum*. From this study, it can be concluded that isolate $\mathbf{Y_{16}}$ showed the highest antagonistic activity than the other yeast isolates and *Penicillum italicum* is one of the responsible fungi for orange blue mold disease in Ethiopia.

Keywords: antagonist yeasts, biocontrol, *Penicillium italicum,* orange, yeast.

1. INTRODUCTION

Orange (*Citrus sinesis*) is one of the major commercial fruit crops that is widely consumed both as fresh fruit or juice. Its global demand is attributed to its high vitamin C content and its antioxidant potential (Gorinstein *et al*., 2001). Orange is mainly cultivated in the subtropical and tropical regions of the world between 40^0 north and south latitude in over 137countries (Ismail and Zhang, 2004). Brazil is the largest producer followed by the United States of America (USA), China and Mexico. Spain, USA and South Africa are the largest exporter countries followed by Turkey and Morocco (Citrus Commodity Notes, 2005).

Orange production in the world was estimated to be 68.3 million tones in 2010 (FAO, 2012). Orange production in Africa was estimated to be above 4 million tones in 2010 and the major producers were Egypt, South Africa and morocco. Egypt is leading orange producing country in Africa. In Ethiopia orange is mainly cultivated in eastern and western part of the country (FAO, 2012). Green molds which is caused by *Penicillium digitatum Sacc* and blue mold which is caused by *Pencilim italicum. Wehmer* are the main wound pathogen post harvest diseases of orange fruit. They are the most common and the most devastating postharvest diseases. They are economically important and occur in all citrus growing countries and may attack the fruits in packinghouses, in transit, in storage and in the market (Barkai-Golan, 1966).

According to a 1965 U S Department of Agriculture survey, post harvest losses in fruits, nuts, and vegetables amounted to about 23% of the harvested crop (Pusey and Wilson, 1985).Losses in developing countries run even higher because of poor storage and food

handling technologies. Post harvest losses in tropical Africa and India have been put at 30% (NAS, 1987).postharvest losses of perishable (vegetable and fruits) food crops in Ethiopia amounted about 30-50% (EARO,2003). Although several management strategies have been developed to control quiescent infections, they often involve the extensive use of fungicides, which are both expensive for growers in developing countries and potentially damaging to the environment and also its toxicity risk (Dodd *et al*., 1989). Thiabendazole (TBZ), imazalil, and benomyl are the most commonly used postharvest fungicides for oranges. However, the excessive pre- and post-harvest use of this chemical has led to a reduction in its effectiveness due to development of resistance in the pathogen population (Spalding, 1982)

So, alternative methods of control are needed because of the negative public perceptions about the use of fungicides among fungal pathogens, and high development costs of new chemicals. In recent years, biological control of postharvest diseases of fruits has become an important field for research. A number of yeasts and bacteria have been report to inhibit postharvest decay of fruit effectively (Janisiewicz and Korsten, 2002).

2. Objective of the study

2.1. General objective

To isolate and characterize indigenous biocontrol yeasts occur on Phyllospher of orange, apple, mango and avocado plants for their antagonistic activity against the growth of orange blue mold fungi.

2.2. Specific objectives

- ✓ To isolate, characterize and identify orange blue mold fungi from infected orange fruit.
- ✓ To isolate, characterize and identify biocontrol yeast which have potential to control the development of orange blue mold disease.
- ✓ To evaluate the potential of antagonistic yeasts of orange blue mold development on harvested orange fruit.

3. LITRATURE REVIEW

3.1. Orange

Orange (*Citrus sinesis*) is one of the major commercial fruit crops that is widely consumed both as fresh fruit or juice. Its global demand is attributed to its high vitamin C content and its antioxidant potential (Gorinstein *et al*., 2001). Orange is mainly cultivated in the subtropical and tropical regions of the world between 40^0 north and south latitude in over 137countries (Ismail and Zhang, 2004).

3.2. Global orange production and utilization

Like most citrus plants, oranges do well under moderate temperatures between 15.5 and 29 °C (60 and 84 °F) and require considerable amounts of sunshine and water. It has been suggested that the use of water resources by the citrus industry in the Middle East is a contributing factor to the desiccation of the region. Another significant element in the full development of the fruit is the temperature variation between summer and winter and, between day and night. In cooler climates, oranges can be grown indoors (Ismail and Zhang, 2004).

Brazil is the world's leading orange producer, with an output almost as high as that of the next three countries combined (the United States, India, and China). Orange groves are located mainly in the state of São_Paulo, in the southeastern_region of Brazil, and account for approximately 80% of the national production. As almost 99% of the fruit is processed for export, 53% of total global frozen_concentrated_orange_juice production comes from this area and the western part of the state of Minas Gerais. In Brazil, the four predominant orange varieties used for obtaining juice are Hamlin, Pera Rio, Natal, and Valencia. United States is the second largest producer. Groves are located especially in Florida, California, Texas, and Arizona. The majority of

California's crop is sold as fresh fruit, whereas Florida's oranges are destined to juice products. Mid-south Florida produces about half as many oranges as Brazil, but the bulk of its orange juice is not exported. The Indian River area of Florida is known for the high quality of its juice, which often is sold fresh in the U.S. and frequently blended with juice produced in other regions because Indian River trees yield very sweet oranges (Citrus Commodity Notes, 2005). Oranges, whose flavor may vary from sweet to sour, are commonly peeled and eaten fresh or squeezed for juice. The thick bitter rind is usually discarded, but can be processed into animal feed by desiccation, using pressure and heat. It also is used in certain recipes as a food flavoring or garnish. The outermost layer of the rind can be thinly grated with a zester to produce orange zest. Zest is popular in cooking because it contains the oil glands and has a strong flavour similar to that of the orange pulp.

The white part of the rind, including the pith, is a source of pectin and has nearly the same amount of vitamin C as the flesh and other nutrients. Although not so juicy or tasty as the flesh, orange peel is edible and has higher contents of vitamin C and more fibre. It also contains citral, an aldehyde that antagonizes the action of vitamin A, Particularly in environments where resources are scarce and therefore maximum nutritional value must be obtained with the minimum generation of waste, for example, on submarine, orange peel have been consumed routinely. Since large concentrations of pesticides have been found in orange peels.Some organizations recommend consumption of the peel of only organically grown and processed oranges, where chemical pesticides or herbicides would not have been used Gorinstein *et al*., (2001).

Orange blossoms are used in several different ways, as are fruit peels and the leaves and wood of the tree. Orange blossom essence is an important component in the making of perfume. It can also be made into a delicately citrus-scented version of rosewater, known as "orange blossom water" or "orange flower water". It is a common ingredient in French and Middle Eastern cuisines, especially in desserts and baked goods. In some Middle Eastern countries, drops of orange flower water are added to disguise the unpleasant taste of hard water drawn from wells or stored in qullahs (traditional Egyptian water pitchers made of porous clay). In Spain, fallen blossoms are dried and used to make tea. Orange blossom honey (or citrus honey) is obtained by putting beehives in the citrus groves while trees bloom. By this method, bees also pollinate seeded citrus varieties. This type of honey has an orangey taste and is highly prized. Marmalade usually is made with Seville oranges.

All parts of the fruit are used: the pith and pips (separated and placed in a muslin bag) are boiled in a mixture of juice, slivered peel, sliced-up flesh, sugar, and water to extract their pectin, which helps the conserve to set. Orange peel is used by gardeners as a slug repellent. Orange leaves can be boiled to make tea. Orangewood sticks are used as cuticle pushers in manicures and pedicures, and as spudgers for manipulating slender electronic wires and also it is used in the same way as mesquite, oak, and hickory for seasoning grilled meat (Libman, and Trakhtenberg, 2001).

3.3. Nutritional value of orange

Energy	197 kJ (47 kcal)
Carbohydrates	11.75 g
Sugars	9.35 g
Dietary_fibre	2.4 g
Fat	0.12 g
Protein	0.94 g
Vitamin_C	53.2 mg
Vitamin_E	0.18 mg
Calcium	40 mg
Iron	0.1 mg
Magnesium	10 mg
Manganese	0.025 mg

(Libeman and Trakhtenberg, 2001).

3.4. Orange production in Ethiopia

Commercial fruits like banana, mango, avocado, orange, papaya and apple are believed to have been introduced into Ethiopia by traders, religious groups and foreign powers. Most indigenous fruits are wild. Large commercial fruit production, banana and orange in particular, flourished during Emperor Haile Sellassie's time. Orange is among the most important citrus fruit crops of Ethiopia. Its cultivation started in Upper Awash valley and Melkassa areas in southeast Ethiopia. Upper Awash eco-conditions proved best for orange, mandarin, tangor and tangelo, while middle Awash was appropriate for grape fruit, lemon and lime (Herath *et al*., 1994).

The Rift Valley, including the Upper Awash and the lake region in Eastern Showa; the Amhara region that covers the Lake Tana catchments;(Bahirdar Zuria, West Gojam and South Gondor) SNNPR: Awasa (Sidame), Arbaminch and Chencha highlands.Dire Dawa and the Tigray region of the Axum-Adowa belt, are currently the main areas for orange production in Ethiopia,

according to a study by the Ethiopia horticulture production exporters association (EHPEA), They have the potential to produce up to 150,000tn of oranges. Sweet orange cultivation covers 82% (1 732.51 ha) of the total citrus area surveyed in the country (In total, production and area harvest of citrus has increased in Ethiopia by 35.1% (Sissay Bekele, 2007).

Oromia: areas around Addis Abeba (Eastern Shewa, Northern Shewa, Western Shewa), including some of the major floriculture production areas. Citrus occupied 7290 hectares of land with production of 230,970 m tones in 1985. Out of the 12 varieties of orange that are grown in Ethiopia, only Valencia oranges are produced throughout the year.

However, its production rate is minimal compared with other African countries. In 2010/11, Ethiopia exported 8,324tn of orange to Djibouti and Sudan earning 2.8 million dollars, according to data from the Ethiopian Revenues & Customs Authority. The country produced 439,792tn of orange in the same year. The total sweet orange produced in Ethiopia is currently almost sold as whole fruit, with out further processing or preparing into other forms. A very insignificant amount of it is used for preparing frozen and canned orange juice, extracts and preserves (FAO, 2004). Data from the Ethiopian Revenues & Customs Authority (ERCA) indicates that orange exports, since 2005, have gone almost exclusively to Djibouti, with a small amount going to the Sudan and the United Arab Emirates. Between 2005/06 and 2010/11 fiscal years, total annual exports ranged from 1,811 ton to 8,324 ton, with respective revenues between one to 1.5 million dollars. This data indicates that orange production and export become increasing and also Ethiopia start getting foreign currency from orange production.

3. 5. Importance of post harvest losses

Postharvest diseases that cause spoilage of both durable and perishable commodities are widespread. Greater losses occur in developing countries due to non availability of proper storage and transportation facilities and improper handling methods, resulting in greater levels of injuries or wounds during harvesting and transport. The harvested produce might have been infected by pathogens prior to harvest under field conditions or they may get infected during transit and storage (Waller, 2002). Harvested food has a higher value than the same crop in the field. A harvested food Commodity carries the cumulative cost of soil preparation, planting, fertilization, watering, pest and weed control, harvesting and distribution. Therefore postharvest loss of a high value commodity has a tremendous impact on the total food production budget (Pusely and Wilson, 1985). Losses may be both quantitative and qualitative. Loss caused by postharvest diseases may be greater than the economic gains achieved by improvements in primary production. Studies on postharvest diseases are primarily directed at preventing economic loss from spoilage of harvested commodities during transit and storage, and at eliminating the adverse effects of mycotoxins produced by fungal pathogens contaminating both durables and perishables. The mycotoxins are known to be carcinogenic, causing several serious ailments in humans and animals (Narayanasamy, 2005). Post harvest diseases of fruits and vegetables are a major expense in food production.

Losses are difficult to estimate reliably, but according to a 1965 U.S. Department of Agriculture survey, post harvest losses in fruits, nuts, and vegetables amounted to about 23% of the harvested crops (Pusey and Wilson, 1985). Losses in less developed countries run even higher because of poor storage and food handling technologies. Post harvest Losses in tropical Africa and India have been put at 30% (NAS, 1987).

3.5.1. Post harvest loss of orange and its management in Ethiopia

Postharvest losses of perishable (vegetable and fruits) food crops in Ethiopia amounted to about 30-50%. Postharvest Losses of orange in Ethiopia is estimated about 9%. Green and blue mold are the most economically important and devastating groups of post harvest disease of orange. High moisture content, insect infestation and fungal spoilage are the major causes of post harvest loss of fruits in Ethiopia. Poor handling Practices, lack of cool storage facilities and insufficient postharvest treatments are the most important reasons of postharvest loss(Eyob,1997).

Preharvesting (individual peasants) and cool chain management (exporters) are the most common methods used to control post harvest losses of fruits and vegetables in Ethiopia (EHDA, 2008).

3.6. Post harvest diseases of orange fruit

Harvested orange are susceptible to invasion by certain pathogenic fungi and bacteria since their high moisture levels and nutrients are conducive for microbial growth. There are many fungi that attack orange fruit after harvest. Among these fungi, *Penicillium digitatum Sacc* Causal organism of green mold and *Penicillium italicum Wehmer* responsible organism of blue mold are the main wound pathogens of orange. They are the most common and the most devastating postharvest diseases of orange fruit. They occur in all citrus growing countries, worldwide and may attack the fruits in packinghouses, in transit, in storage and in the market.

Conidia of the Penicillia are present during the season in the atmosphere of citrus growing areas, particularly in citrus packinghouses and their surroundings, on the packinghouse equipment or on the hands of selectors and packers (Barkai-Golan, 1966). Stem-end rots caused by *D, natalensis* and *Penicillium citri* are the major postharvest diseases of orange Infection may be initiated at any stage of fruit development, when wind and splashes of rain carry the pathogen spores to the surface of immature fruits on the tree. However, immature fruits are resistant to invasion, and the fungi remain quiescent in floral remnants under the sepals of the fruit and do not become active until the buttons become senescent and begin to separate from the fruit (Brown, and Wilson, 1968). Brown rot which is caused by *Phytophthora* species are major post harvest disease of orange fruit .It is particularly prevalent after late-season rains, which play an important part in disease development. The fungi are common in soil and survive in their sexual state as thick-walled oospore (Houg *et al*., 1980). Although *Colletotrichum gloeosporioides,* the causal fungus of anthracnose and *Trichoderma viride* the causal organism of Trichoderma rot are not included in the list of the major pathogens in orange fruit, they may be a serious disease when fruits harvested early in the fall (Brown,G.E.,1975; Gutter, 1961).

3.6.1. Orange blue mold

3.6.2. Causal organism

Orange blue mold is caused by fungi known as *penicillium italicum.* It is the most economically important and devastating wound pathogen organism. This disease attacks all citrus fruits and occurs in all citrus growing countries, world wide (BarkaiGolan, 1966).

3.6.3. The infection process

Conidia of the Penicilliu species (*P.digetate and P.italicum*) are present during the season in the atmosphere of citrus growing areas, particularly in citrus packinghouses and their surroundings, on the packinghouse equipment or on the hands of selectors and packers (Barkai-Golan, 1966). The disease is characteristically initiated through wounds and mechanical injuries sustained during harvesting, packing and handling (Kavanagh and Wood, 1967).Wounds may also result from piercing by the Mediterranean fruit fly and other fruit-piercing insects (Roth, 1967). Fruits are particularly susceptible to infection during wet or humid weather, and the postharvest temperature is another factor determining the blue mold fungi development. The optimal temperature range for this fungi is 20-27°C, within which the fruits may rot within a few days. Although fungal growth is reduced at lower temperatures, a very slow rate has still been recorded at 4.5-10°C, allowing the fungi to progress under these conditions when storage is extended or in overseas shipments. At 0-l°C the growth of Penicillia is arrested, but these temperatures result in chilling injury, expressed in pitting and internal physiological injury (Smoot *et al*., 1983).

3.6.4. Symptoms

At an early stage the fungi cause a soft rot of the peel. Following this stage, a white mycelium develops from the center of the affected soft area; it later starts sporulating from the center of the colony, which is the older part of the infected area. The sporulating part becomes olive blue.The rot at this stage is characterized by three circles. The colored sporulating center (1) is surrounded by (2) a band of white mycelium which has not yet sporulated or has only just begun to form conidiophores, or conidiophores plus sterigmata (which will later bear the spores). This white band is surrounded in turn by (3) a definite band of water-soaked peel, which has not yet

developed the white fungal mycelium. Softening of the peel tissue is associated with the activity of pectolytic enzymes produced by the pathogen during pathogenesis. Under dry conditions the decayed fruit shrinks and becomes 'mummified (Stange and Eckert, 1994).

3.6.5. Control of blue mold after harvest

The possibility of penetration of the wound pathogens into the fruit via injuries in the peel shows why the control of insects in the grove and careful handling throughout the harvesting process, to prevent skin breaks and bruises, are of primary importance in preventing the development of infections. The severity of postharvest wound infections is also directly related to the damage caused to the crop by rough handling after harvest (Sommer, 1982).

Thus, careful handling should always be a continuous concern; the fact that preharvest fungicide sprays in the grove have not always been effective in reducing postharvest decay is probably because most injuries occur during harvesting or packinghouse handling. Sanitation to minimize the presence of infective inoculum is also great importance for disease prevention. It includes the removal of fallen or decayed fruit from the grove and from the packinghouse and its surroundings, sanitation of packinghouses by fumigation (with formaldehyde or other fumigants), sanitation of field boxes with disinfecting solutions or steam, and unloading and cleaning fruit arriving in the packinghouse in a separate area, to reduce contamination in the processing, packing and storage areas (Smoot *et al*., 1983).

In addition to sanitation procedures, strategies for control of postharvest diseases incited by wound pathogens include: (a) inactivation of germinating spores in fresh wounds; (b) protection

of the peel from infection at a later time by depositing a fungitoxic residue in wounds or on the surface of the fruit; and (c) inhibition of *Penicillium* sporulation on the surface of decaying fruits and of subsequent contact spread in storage (Eckert, 1990). Healing of wounds and control of postharvest decay has also been achieved by combining individual wrapping of citrus fruit in plastic film, which leads to the formation of a water-saturated atmosphere within the wrap, with curing at 36°C for 3 days (Benyehsoshua *et al*., 1987). Storing oranges between 2°C to 3°C (36°F to 38°F) is optimal post harvest temperature. Market life at this temperature range will be up to 4 months, depending on cultivar and maturity stage at harvest (Smoot *et al*., 1983). Much research has been conducted on the development of biological control means as alternatives to chemical treatments, for controlling postharvest decay (Wilson and Wisniewski, 1989).

Chalutz and Wilson (1990) found that yeast species of the genera *Debaryomyces* and *Pichia* are capable of inhibiting wound pathogens because of their rapid development in wounds in citrus fruit peel. Arras (1996) found that *Candida famata* was one of the most active yeasts against P. *digitatum* in wounded fruits, while Smilanick and DenisArrue (1992) and Smilanick et al. (1996) demonstrated that strains of the bacterium *Pseudomonas syringae* could reduce the incidence of postharvest rot by occupying the wounds. Isolates of the common yeast-like fungus, *Aureobasidium pullulans* were reported by Schena *et al*. (1999) to control P. *digitatum* at high concentrations on grapefruit. Recently, two biological products have been registered for commercial postharvest applications to citrus fruits - **Aspire,** which is *Candida oleophila,* and **BioSave"**1000, which is P. *syringae* (Brown and Chambers, 1996).

3.7. Post harvest biological control of fruit diseases

There is a renewed interest in improving production through use of environment friendly products such as bionoculants, instead of chemicals. This approach may ensure that nature is not exploited in the production process but is instead harmonized so that sustainability in agricultural production is promoted (Narain, 1998). Although the concept of using biocontrol agents to increase plant yield goes back over 100 years, relatively very few reliable bionoculant products are currently available on the market. This is mainly because of the lack of confidence among the end users about the performance of the products being marketed (Nsutiyal, 2000).

Although biological control occurs naturally and is the principal reason that diseases are not catastrophic in natural ecosystems, in most cases sufficient knowledge is not available to explain how biological control operates naturally or how the multi-faceted abiotic and biotic factors can be manipulated to effect economic control of a pathogen (Nautiyal, 2000). As a result, as stated by Schroth and Hancock (1981) biological control of plant pathogens is a fascinating, challenging, but elusive and frustrating area of study for plant scientists and microbiologists. Research into controlling plant pathogens by biological means has mostly been directed to manipulation of the environment to favor the introduced antagonist in the appropriate ecological niche so that it will be active against the pathogen. The overwhelming problem in the area of biocontrol agents is to get repeatable results particularly in the field. When taken outside the laboratory or green house, antagonists fail to show effects which are consistent from year to year and over different climatic and soil types (Jeyarajar and Kakkeeran, 2000).

Biocontrol often requires the introduction and establishment of an organism or organisms in the microbial community on or near the plant surface. The mechanism of disease control must be appropriate to the plant, the pathogen, the site of activity and the development stage of the target

organism. A massive research effort on population biology and mechanisms of interactions of biocontrol agents and the pathogen is required. Among the several types of microorganisms i.e. bacteria, fungi, and viruses, bacteria have been used most extensively against plant pathogens. Bacterial antagonists are popular for several reasons, including the relative ease of isolation, growing, identification, tracking, genetic manipulation, and laboratory and commercial applications (Jeyarajar and Kakkeeran, 2000).

3.7.1. Opportunities for biological control of post harvest plant disease

Antagonistic microorganisms for epiphytic biocontrol often are effective in petri dishes or in the green house but fail in the field. Leben *et al.* (1965) indicate that destruction by ultraviolet rays and dessication are major reasons for such failure. Storage conditions for food commodities do not present the same hazard for antagonists (Pusey and Wilson, 1985). In the developing countries losses of perishable products are particularly high due to lack of refrigeration and proper sanitation. Biological control offers great scope for management of postharvest diseases of fruits and vegetables due to several reasons. Many fruits and vegetables are stored under controlled environment and area ofapplication harvested for fruits and vegetables is less than in the crop field and orchard, and it is easier to apply the biocontrol agents to target site. Moreover, high value of fruits makes biocontrol agents more cost effective, and biocontrol agents are not much exposed to UV radiation (Jeyarajar and Kakkeeran, 2000).

As food commuters mature in the store they became “leaky” nutrients that favor the growth of microorganisms which became bountiful on the surfaces. This nutritional milieu favors development of rot pathogens but may also favor development of antagonist or parasites of the

pathogen. Competition for nutrients in a particular biological niche is an important form of biocontrol (Cook and Baker, 1983). During storage period yeasts may play a crucial role in the management of post harvest diseases. Yeasts are able to colonize the fruit surface for long period under dry conditions and they produce extracellular polysaccharides that enhance their survival and restrict flow of nutrients to pathogen propagules. Rapid use of available nutrients and proliferation is the other quality of yeasts. They are also least affected by pesticides and are good competitors and do not produce toxic metabolites (Jeyarajar and Kakkeeran, 2000).

3.7.2. Application of biocontrol agents against post harvest diseases

With all these opportunities for post harvest biocontrol, what has actually been done is surprisingly little (Pusey and Wilson, 1985). In apple, fruit dip with a suspension of *Cryptococcus laurentii* (10^7 spores or cells/ml) gave effective control of *Botrytis cinerea* during cold storage. In strawberry, Peng *et al*. (1992) found that spraying a conidial suspension of *Trichoderma viride* (10^7/ml) from early flowering at 14 days interval reduced fruit rot (*Botrytis cinerea* and *Mucor mucedo*) during storage comparable to the fungicide dichlorofluanid. Lim and Rohrbach (1980) were able to reduce the incidence of disease in pineapple fruit caused by *Penicilium funiculosum* by spraying the fruit with non-pathogenic strains of the pathogen. Most applications of antagonists to control post harvest diseases have been made before harvest (Pusey and Wilson, 1985).

Post harvest applications have not been fully exploited. Post harvest applications can be done by spraying at a given rate over the fruit or by dipping in a certain concentration of the cell/mycelial suspensions of the antagonist. Mixing antagonists with commercial wax formulation and applying to fruit surface was found effective. When an antagonist that is resistant to a certain fungicide is applied with the fungicide, the antagonist because of its resistance can be more

dominant and effective (Pusey and Wilson, 1985). Once a biocontrol procedure has been developed, its application will need to be integrated with the existing pest control programs.

4. MATERIALS AND METHODS

4.1. Isolation of fungal pathogen

Orange blue mold causing organism was isolated from orange fruits showing Blue mold lesion which were obtained from fruit shops and markets in Addis Ababa. The spore was taken from infected part of orange fruit by cutting the fruit peel in to pieces and sterilized using 70% alcohol then inoculated to malt extract agar plate. After incubation of malt extract agar plates for 7days at 25^0c, developed colony was characterized based on its gross morphology and microscopic examination, then it was transferred to fresh malt extract agar plates and the pure culture grown on malt extract agar was maintained at4^0c as stock culture to use it for in vivo and in vitro test (Ibrahim and Rahma, 2009).

4.2.Isolation of the antagonistic yeasts

Antagonistic yeasts were isolated from the phyllospher of health looking apple, orange, avocado and lemon, mango plants which were sampled from market and unmanaged

trees in different ecological regions and habitats of Ethiopia (Addis Ababa, Holleta, Sebeta, Jimma, Agaro and Chenchea).Samples which were separately put in sterilized plastic bags were taken to the Mycology Laboratory of AAU for isolation of potential antagonistic microorganises. Sub-samples of fruit peels and leave cut pieces 10g of each were shaken in 90 ml of sterile distilled water for 10 min on shaker.

Aliquots of the samples were washed and inoculated on malt extract agar plates which were amended with tetracycline by spread plating method and incubated at 25^0c for 3-days then the developing colonies were characterized based on their gross morphology and microscopic examination. Representative isolates were transferred to fresh malt extract agar plates and pure cultures were maintained at 4^0c until they use for in vivo and in vitro test (Wilson and chalutz,1989).

4.3. Characterization and identification of the pathogen

The characterization and identification of the pathogen that cause orange blue mold disease was done based on their macroscopic (colony color, size, texture, pigment production and margin) and microscopic morphological characteristics (form of hyphea, shape, type and color of spores) as well as by their biochemical character (growth character on various temperature and media). (Pitt, 1979).

4.3.1. Morphological characterization of the pathogen

4.3.1.1. Macroscopic characterization of the pathogen

In order to investigate macroscopic morphological characteristics, a suspension was prepared from spores removed with a sterile loop from sporulating edges of a 1 week-old culture and suspending them in 1ml Sterile distilled water. Petri dishes (90 mm) containing PDA (which was made in triplicate) were inoculated with the pathogen at the center of the plates and then all plates were incubated at 25^0C for 7 days. Diameter, margin, color and texture of the colony were recorded.

4.3.1.2. Microscopic characterization of the pathogen

Slid culture method has been done to study microscopic features (branching of hyphea, conidium shape, color and length) of the pathogen.

Sheet of sterile filter papers were placed in sterilized Petri dish using sterilized forceps then sterile U-shaped glass rods were placed on the filter paper. Sterilized water was poured (about 4 ml) on filter paper to completely moisten it. Then sterilized slides were placed on the U-shaped glass. 5 mm square blocks of the medium (SDA) were cut using sterilized scalpel and then blocks of saburuad were picked up by inserting the scalpel and carefully transferred to the center of slides. Four sides of the agar squares (blocks) were inoculated with spores of the pathogen. The upper surface of the agar cubes were covered with sterilized cover slip. Then the plates were incubated at 25^0C for 7 days. After 7-days, microscopic examination has been done and appearance of hyphae, shape, color and length of conidium were recorded (Wijedaso and Liyanapathirana, 2012).

4.3.1.3 Biochemical characterization of the pathogen

The growth characteristics of the pathogen at different media (potato dextrose agar Malt extract agar, saburaud dextrose agar and yeast peptone dextrose agar) and temperature was also studded in this work.

Petri dishes (90 mm) containing saburaud dextrose agar, potato dextrose agar, malt extract agar and yeast peptone extract agar (each were prepared in triplicate) were inoculated with plug of the pathogen (3mm) at the center of the plate. Cultures were incubated at 25^0C for 7 days and morphology of the colonies was recorded. To investigate the growth characteristics of pathogen at different temperature, the pathogen was inoculated on potato dextrose agar and incubated at,20^0C, 25^0C, 27^0C, 30^0C and 37^0C for 10-days then colony diameter, margin, color and texture of pathogen was recorded.

4.4.Screening for antagonistic activity (in vitro)

All yeast isolates which were obtained from the samples have been tested for their antagonistic activity against orange blue mold growth. Screening of yeast isolates for their antibiotic production were tested based on inhibition of growth of orange blue mold causing organism by inoculating yeast isolates and the pathogen in the same culture plate. Control plates were inoculated with the pathogen alone.

4.4.1. Testing the potential of antagonism

Mycelia plug (3mm) of the seven day old pathogen was taken by using cork borer and placed at the center of the plate (9mm) then loop full (10^8 cell ml^{-1}) of yeast isolates were inoculated at four nearly equidistant points 3 cm away from the pathogen and incubated for 10 days at 27 ^{0}C. Fungal growth was determined by measuring the colony width at two points using meter ruler, and determine the mean. Pathogen growth on treated media was compared with the control. Three plates were used per-treatment and each experiment was repeated twice. Data obtained were statically analyzed.

The antibiotic activity of the isolates was evaluated using the method described by Mc keen *et al*. (1986). Yeasts which show antagonistic activity against orange blue mold development in vitro were also tested to evaluate their antagonistic activity against artificially inoculated orange fruit.

4.5. In vivo testing for antagonistic activity

4.5.1. Evaluation of antagonistic activity on artificially inoculated fruits

Aliquots of 200 µl of the pathogen suspension from 7-days old culture was deposited with a sterile pipette on the orange wound (5mm) immediately after wounding and allowed to dry in aseptic conditions. After pathogen inoculation, 200 µl of a suspension of the potential biocontrol yeasts namely Y_3, Y_{10} and Y_{16} were also deposited on the wound. The inoculated fruits were incubated on a humid chamber at 25ºC, to simulate the best conditions for the growth of the pathogens, infected oranges were scored after 12 days, respectively, after inoculation. All treatments were repeated three times. To test the ability of potential yeast isolates to control orange blue mold development on oranges, inoculation with same amount of pathogen alone served as a positive control (Raphael *et al*., 2008).

4.5.2. Assessment of lesion

The percentage of disease severity reduction (DSR %) was calculated by the equation: DSR (%) = [(DSC - DST)/DSc] X 100, Where DSc = average area with lesions on the positive control and DSt = area with lesions on the treated orange (2). Only the mechanically wounded region of the orange was used for the assessment of disease reduction.

4.6. Characterization and identification of Antagonists

The characterization and identification of yeasts that showed antagonistic activity against orange blue mold development was done based on their macroscopic (colony color, shape) and microscopic morphological characteristics as well as by their physiological and biochemical features (fermentation test, ascospore formation and growth character on various temperature and media respectively.).

4.6.1. Morphological characterization

Morphological characteristics of the potential yeasts were studded based on the method of Kreger-van Rij (1984) and Kurtzman and Fell (1997). The vegetative cells were grown in liquid and on solid media. Yeast cells were observed microscopically to examine their cell size, shape and how they reproduce (by multilateral, bipolar or unipolar budding, by fission, by forming filaments), and colony color.

4.6.1.1 Growth on solid medium

The macroscopic features of potential yeast isolates were investigated by growing them on MEA after incubating at 27°C for 3 days. The following cultural features of the isolates were recorded; texture, size, margin, color and surface of colonies.

4.6.1.2. Growth in liquid medium

Yeast cells from a young actively growing culture were inoculated into test tube containing 7 ml of medium (malt extract broth) and incubated at 30 °C for 3 days. The shape of cells, and pseudo mycelium formation and their vegetative reproduction was determined using compound microscope.

4.6.2. Physiological characterization

4.6.2.1. Ascospore formation

The potential yeast isolates were examined for ascospore formation according to Kurtzman *et al*. (2005). Accordingly, two types of media were prepared, i.e. pre sporulation and sporulation media. Pre sporulation media was composed of 10g of glucose, 1g of ammonium sulfate ($(NH_4)_2SO_4$), 1g of potassium dihydrogen phosphate (KH_2PO_4), 2.5g of yeast extract and 500ml of distilled water were kept in sterile state for 7 days. The media was inoculated with a loopful of young culture of 48hrs old antagonistic yeast isolates and then incubated at 25^{o}C on shaker for 3 days. The sporulation medium, which was made from, 0.5g of glucose, 4.1g of potassium

acetate, 1.25g of yeast extract, 0.93g of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) and 500ml of distilled water, was inoculated with a drop of yeasts from the presporulation media.

In contrast to the presporulation medium, this medium was used immediately after sterilization. The inoculated media were further incubated at 25^oC to induce ascospore formation. The culture was examined microscopically for ascospores production at weekly intervals for 3 weeks.

Ascospore formation was detected by staining the heat-fixed preparation (Kreger-van Rij, 1984) carbol-fuchsin after have been steamed gently 5 min. Slide was decolorized with 95% ethanol containing 1% concentrated hydrochloric acid. The slide was rinsed in water and counter stained with 1% methylene blue; the mature ascospores stain red and vegetative cells blue.

4.6.2.2. Fermentation of carbohydrates

The fermentation of carbohydrates by yeasts was detected by using the method of Barnett *et al*. (2000). The ability to use 6 kinds of carbohydrates an aerobically, i.e. glucose, sucrose, maltose, lactose, starch and galactose was assessed by looking for the formation of gas (CO_2). Large Durham tubes were used: test tubes of about 150mm by 12mm with insert tubes of about 50mm by 6mm. The tubes were filled with 10ml of YPD broth (yeast extract, 10g; peptone, 20g; glucose, 20g; tap water 1,000ml). Each tube was inoculated with a drop of a fresh yeast suspension of YPD broth, taken from an actively growing culture. The tubes were incubated at 25^oC for about 1 week, each day they were shaken, to help sediment the yeast, and examined for bubbles of gas in the inserted tubes (Barnett *et al*., 2000).

4.6.2.3. Effect of Temperature on the growth of antagonists

In order to investigate the growth characteristics of potential yeasts at different temperature they were inoculated on malt extract agar and incubated at 18^0c, 20^0c, 25^0c, 27^0c, 30^0c, 35^0c, 37^0c, 40^0c and 45^0c and their growth condition was recorded.

4.7 Statistical Analysis

Data analysis was carried out using the SPSS software package (SPSS 15.0, SPSS Inc. Chicago,IL, USA).

5. RESULTS AND DISCUSION

5.1. Characterization and identification of the pathogen

5.1.1. Morphological and physiological characteristics of pathogen

The gross morphological characteristics of orange blue mold on different media namely, potato dextrose agar, malt extract agar, saburuad dextrose agar and yeast peptone dextrose agar at 25°C for 7-days is described below. Morphology of the pathogen culture on different media was variable. Colonies on potato dextrose agar= 30-40mm diam; color, greenish gray to grayish green; surface texture, velutinous(rough); margin entire or irregular; pigment, pale, conidiogenesis heavy, mycelium, white; On malt extract agar= colonies 30-35mmin in diam; color, gray; surface texture ,velutinous (smooth); margin irregular; mycelium, white; conidiogenesis; moderate to heavy; pigment, pale; On saburuad dextrose agar= colony 25-30mm diam; color bluish gray; margin, irregular; surface texture velutinous (rough); mycelium white; conidiogenesis, heavy; pigment, near umber; yeast pepton extract agar= colonies15-20mm diam; color, gray; surface texture, velutinous (rough); mycelium white; conidiogenesis, heavy; margin,

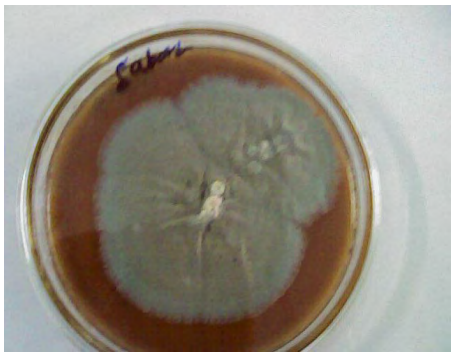
regular; pigment, pale. The growth rate of the pathogen at different media and temperatures has been described bellow (table-1& 2 respectively).

Table- 1: the growth morphology of orange blue mold causing organism on potato dextrose agar PDA, MEA, SDA and YPDA at 25^0c for 7-days.

Type of media	colony					conidia	
	shape	color	Margin	texture	size	shape	color
PDA	circular	Greenish gray	irregular	rough	37mm	ovoid	Grey green
MEA	circular	gray	irregular	smooth	30mm	ovoid	Grey green
SDA	circular	Bluish green	irregular	rough	23mm	ovoid	Grey green
YPDA	circular	gray	regular	rough	16mm	ovoid	Grey grren

This result tell us the growth rate of the pathogen on different media was different ,on potato dextrose agar and malt extract agar it was grow fast and moderate on saboard dextrose agar and yeast peptone dextrose agar and the colony color and surface texture of the pathogen on different media was also different.

A **B**



C **D**

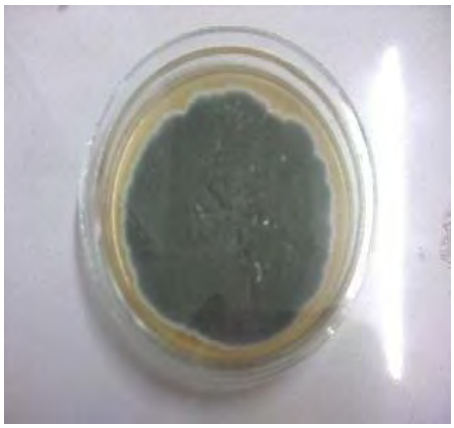


Fig.1: Colony growth characteristics of orange blue mold incubated at 25^0c on different media. (A)SDA, (B) MEA, (C) PDA, (D) YPDA.

tabel -2 the growth rate of orange blue mold incubated at different temprature on PDA for10 days.

Temeprature	Mean of colony diameter of the pathogen in mm.
	mean±SD
20^0c	89.00±1.10[a]
25^0c	79.00±1.23[b]
27^0c	70.00±2.08[c]
30^0c	23.00±1.00[d]
37^0c	-

(-)= no growth

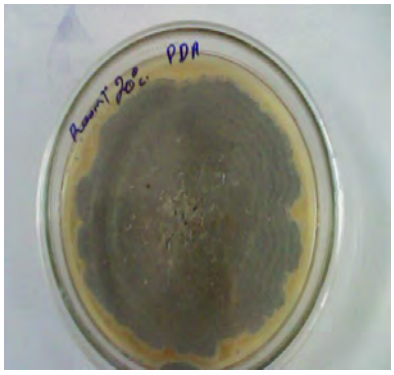
The practical results which were obtaned from this study showes that,the growth rate of ornge blue mold differes at different temprature and on differen media.Based on this result of the study the pathogen grow on potato dextros agar (37mm) followed by malt extract agar (30mm) then sabord dextros agar (23mm) andyeast pepton extract agar (16mm) which was incubated at 25^0c for 7 days. This result tells us orang blue mold

grow best on potato dextros agar and malt extract agar but it has moderae growth on saburaud dextros agar and yeast pepton agar.The growth rate of the pathogen at different temprature was also different. The growth of the pathogen was fast at 20^0c , 25^0c and 27^0c, slow at 30^0c and did not grow at 37^0c within the respective dates.

The result of this study shows that orange blue mold grow best at temprature ranging from 20^0c - 27^0c, the aproperat temprature for development of orange blue mold.

Smoot *et al* (1983) reports that orange become susceptible to blue mold infection during wet or humid weather, and the optima temperature suitable for development of the orange blue mold ranges 20-27°C, with in which the fruits may rot within a few days. This report agrees with the result obtained from this study.

A **B**



C **D**

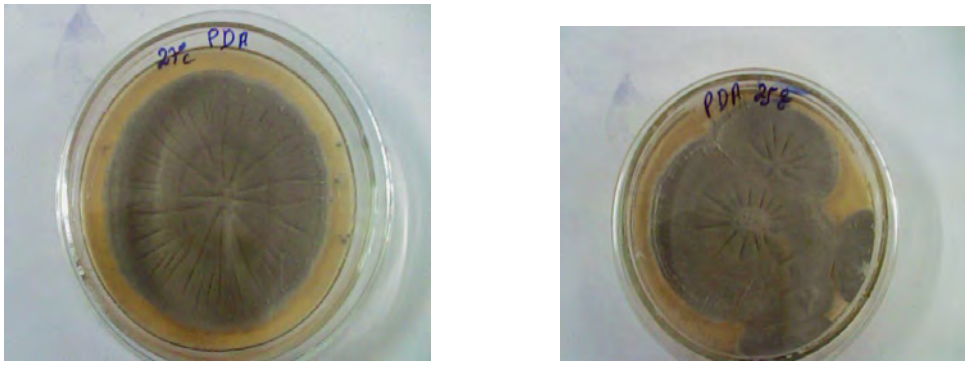


Figure.2: the growth rate of orange blue mold causing organism on PDA at different temperature :(A) 20^0C,(B) 30^0C, (C) 27^0C,(D) 25^0C for 10 days.

The microscopic morphology (penicilus, conidia shape, color and size) of the pathogen was also studded using slide culture technique. The experimental result indicated that the fungal mycelia of the pathogen produce simple, long erect conidiophores that branched about two-third of the way to tip, in a characteristics asymmetrical broom-like fashion. The multiple branching of conidiophores ends in a group of phialides that bear the long conidial chains. The conidia form brush like head, globos and smooth

(Figer-3)

A **B**

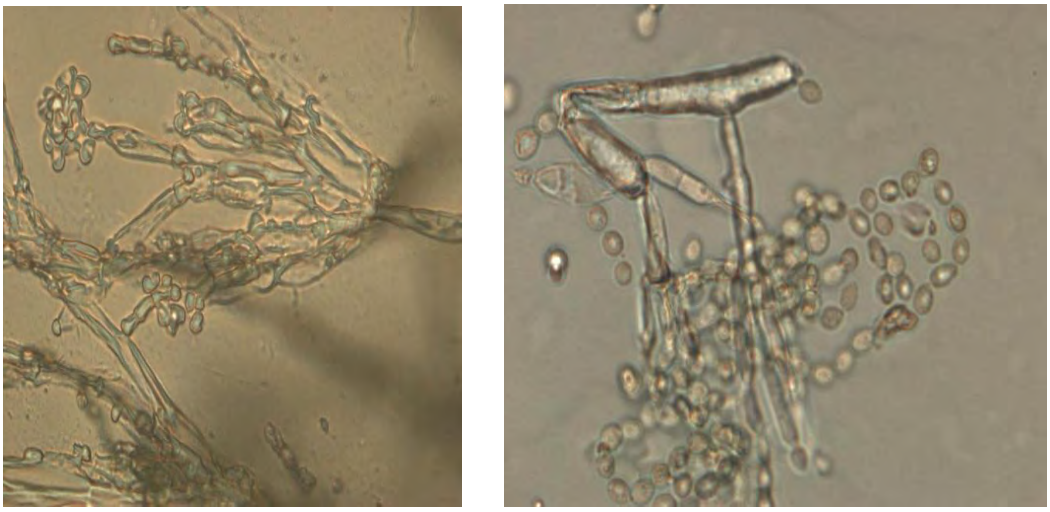


Fig-3: Form of penecilli (A) and conidial shape (B) of orange blue mold examined by light microscope (100x).

In order to investigate whether the pathogen is wound parasite or not artificially wounded and unwounded oranges were inoculated with spores of orange blue mold which was taken from the stock culture of the pathogen. All wounded oranges developed or showed white mycelia rot after three days followed by bluish gray rot with in 7-days unwounded oranges did not developed whitish mycelia rot as well as bluish gray rot. This result tells us orange blue mold causing organism of Ethiopia is wound parasite and production of blush gray or grayish blue rot is symptoms of the disease (Fig4).

Fig-4: Symptoms of orange blue mold disease after inoculating the pathogen on artificially wounded orange fruits.

5.1.2 Identification of orange blue mold causing fungus

Pitt, (1979) has been reported that, the primary habitat for *Penicillium italicum* at present day is fruit of citrus species, on which it produces distractive rot of considerable economic importance. Production of destructive bluish gray rot on citrus fruits and gray green conidia are the distinguishing characters of *Penicillium italicum* from the synonymy *Penicillium digitatum* and also from the other Penicillium species. Results which are obtained from the morphological and physiological characterstics of this study fully agree with the above report.The identity of the pathogen was studed

based on their morphological, physiological and biocheimcal characteristics. Orange blue mold disease causing organism (pathogen) was identified as *Penicillium italicum* (Pitt, 1979).

5.2 Characterization and identification of potential antagonistic yeasts

5.2.1 Morphological characteristics of potential antagonists

The macroscopic and microscopic (cell shape, form of budding) growth characteristics of potential yeast isolates (y_3, y_{10} andy_{16}) were investigated and recorded using different techniques.

All potential yeast isolates reproduced asexually by budding and they do not have sexually reproduction stage or they do not form ascospore.

Colony color, shape, texture, margin, cell shape and surface are shown in tabl-3.

Table-3: Microscopic and Macroscopic growth characteristics of potential yeast isolates on malt extract agar for 72h culture.

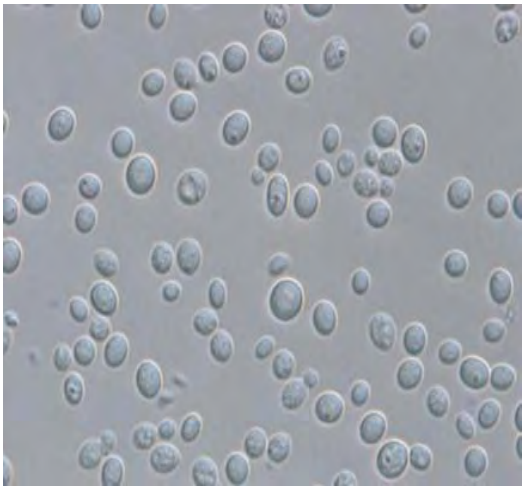
Yeast isolates	colony					cell		
	color	shape	texture	satelight	margin	shape	bud	surface
Y_3	white	irregular	rough	-	irregular	spherical	+	rough

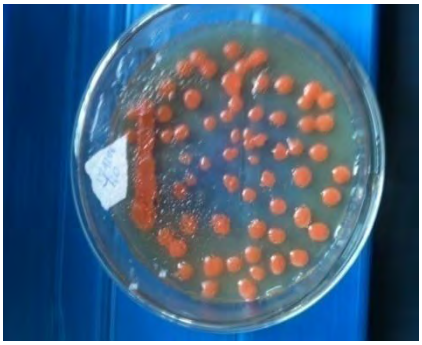
						to ovoidal		
Y_{10}	orange	spherical	smooth	-	regular	ovoid to cylindrical	+	rough
Y_{16}	red	ovoid-cylindrical	smooth	-	regular	ovoidal	+	rough

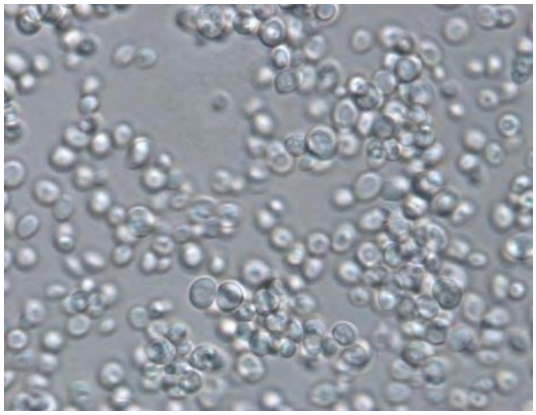
(-) =no sate light colony, (+) = presence of budding

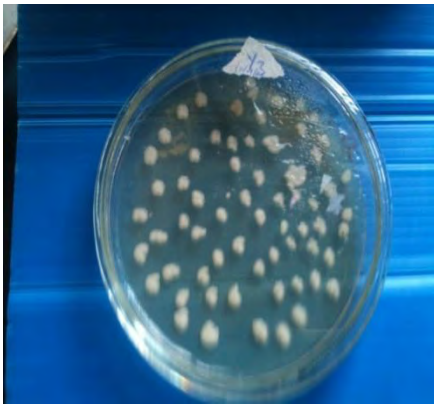
This result tell us all antagonistic yeasts reproduce asexually by budding and lack satelight colony as well as all of them have different colony color and cell shape.

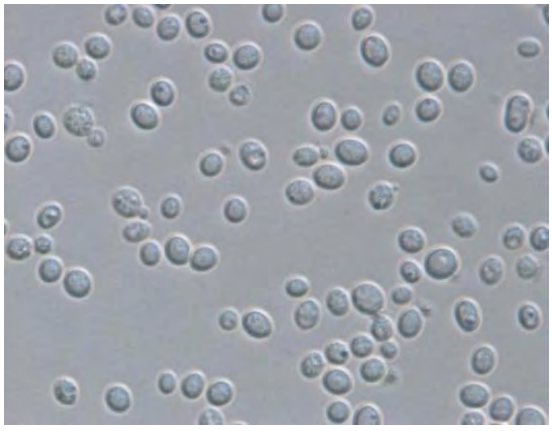
A 

B 



D 

E 

F 

(Fig-5) colony and cell morphology of potential antagonistic yeasts grown on malt extract agar for 3 days (A&B) Y_{16}, (C&D) Y_{10}, (E&F) Y_3 respectively.

5.2.2 Physiological characteristics and identification of antagonists

Physiological properties of the potential antagonistic yeast isolates are shown in Table-4. The fermentative capacity of antagonistic yeasts were tested. Two of potential antagonistic yeast isolates namely y_{10} and y_{16} were found to be negative for the fermentation of glucose, sucrose, maltose, lactose, starch and galactose but y_3 was positive for the fermentation of glucose, maltose, sucrose ,starch and galactose while lactose was not fermented by this yeast isolate. (Table 4). The optimum temperature for the growth of antagonistic yeast isolates has been also investigated in this study which is shown in table- 5.

All potential yeast isolates grow best from temperature ranging from 27^0c up to 37^0c. Based on morphological and physiological Characteristics Y_{10} and **Y_{16}** were identified as Rhodotorula and **Y_3** was belongs to Trichosporonoides. Kurtzman et al. (1997) showed that yeasts which have red to yellow colony color which do not have sate light colony and which do not ferment any one of carbohydrates are identified as Rhodotorula. The morphological and physiological results of y_{10} and y_{16} are vigorously agreed with the above reports.

Tabel-4 Physiological properties of potential antagonistic yeast isolate

Code of yeasts	Fermentation					
	glucose	sucrose	maltose	galactose	lactose	starch
Y_3	+	+	+	+	-	+
Y_{10}	-	-	-	-	-	+
Y_{16}	-	-	-	-	-	+

(+)= positive,(-)= negative

Table-5 the growth of potential antagonistic yeast isolates at different temperature on malt extract agar for 3 days.

Code of antagonists	Growth Temperature							
	10^0c	20^0c	25^0c	27^0c	30^0c	37^0c	45^0c	50^0c
Y_3	-	+	++	+++	+++	+++	+++	+
Y_{10}	-	++	+++	+++	+++	+++	+	-
Y_{16}	-	++	++	+++	+++	+++	+	-

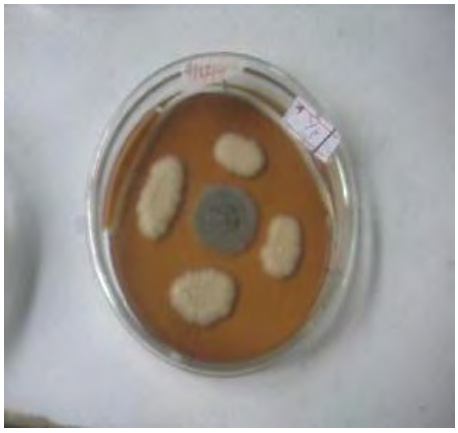
(-)=no growth, (+) =poor growth, (++) =good growth, (+++) = very good growth.

5.3 Inhibition of orange blue mold in vitro

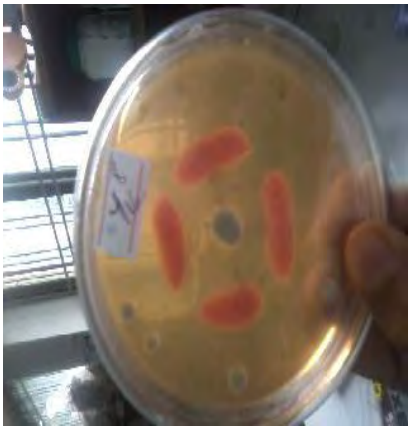
A total of 58 yeasts were isolated from Phyllospher of (leave and fruit surface) mango, avocado, orange, lemon apple plants. All of them were screened *in vitro* to test their inhibitory activity against the growth of orange blue mold. Nine yeast isolates inhibited growth of the adjacent pathogen colony ten days after challenge. Three yeast isolates namely, isolate **Y_3**, **Y10** and **Y_{16}** potentially inhibited the growth of orange blue mold. The level of inhibition of the potential yeasts, isolate **Y_3**, **Y_{10}** and **Y_{16}** was 32%, 36% and 38.7% respectively and all of them significantly reduces orange blue mold development in vitro($P<0.005$). The inhibitory activity was calculated by the Equation (1) reported by Lima *et al.* (1997).

$$\% \text{ Inhibitory activity} = \frac{\text{fungal growth in control plate - fungal growth in treated plate}}{\text{Fungal growth in controlled plate}} \times 100$$

Antagonistic yeast isolates that affected growth of orange blue mold produced distinctly visible inhibition zones. These inhibition zones indicated that production of inhibitory compounds by potential antagonists. The width of inhibition zones largely depends on the degrees of diffusibility and solubility of the inhibitory compounds in the agar media as well as their concentrations. The inhibition zones remained free of pathogen mycelia, however, there is indication of potency and stability of their activity (Fig -6).


A


B


C

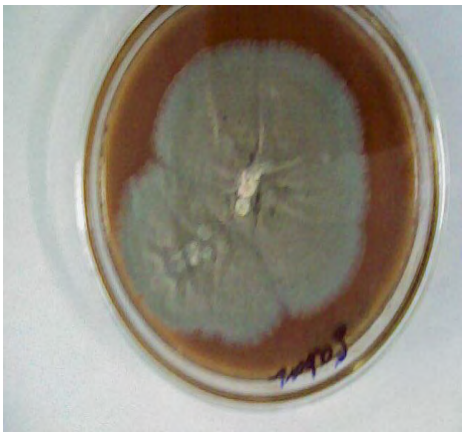

D

Fig-6 Inhibitory activities of potential yeast isolates against the growth of orange blue mold which grow on saborad dextrose agar incubated for 10 days at 25^0c. A= y_3 vs. Pathogen, B=y_{10} vs. pathogen, C=y_{16} vs pathogen, D=pathogen alone (control).

Table-6 inhibitory effect of yeast isolates against in vitro growth of orange blue mold

Code of yeasts	Mean colony diameter of the pathogen Mean ± SD	% of inhibition	rank
Y16	46.00 ± 2.00^{f}	38.7%	1
Y10	48.00 ± 2.130^{df}	36%	2
Y3	51.00 ± 2.40^{de}	32%	3
Y14	54.00 ± 2.00^{cd}	28%	4
Y9	55.00 ± 2.120^{c}	26.7%	5
Y4	57.00 ± 2.20^{bc}	24%	7
Y15	57.50 ± 2.33^{bc}	23.4%	8
Y24	56.00 ± 2.00^{c}	25.3%	6
Y20	60.00 ± 2.00^{b}	20%	9
control	75.00 ± 1.970^{a}	-	-

Yeasts are widely studied as biological control agents and the successful use of the yeast *Rhodotorula minuta* against mango anthracnose has been reported (Patiño-vera *et al*., 2005) as well as Calvente *et al*.(1999) found that *Rhodotorula glutinis* produced rhodotorulic acid, which enhanced biocontrol activity of *Rhodotorula glutinis* against *Penicillium expansum* in postharvest apple. This report tells us the ability of *Rhodotorula* to inhibit apple blue mold and mango anthracnose and other post harvest diseases there fore this report support the result

obtained in our study. Similarly Roberts (1990) discovered that *Cryptococcus laurentii* has antagonistic activity against many postharvest pathogens.

The competition for nutrients may play a role in the antagonism of *Cryptococcus laurentii*. Decay caused by *Rhizopus* sp. is reduced by 70% when strawberries were treated with *Aureobasiduim pullulans* before storage (Lima *et al.*, 1997). Yeasts are known to be abundant in nature on the surface of plants; leaves,fruits and flowers are their most common habitats. They appear to be more adapted than filamentous fungi for coping with fluctuations in ambient water potential that are inherent to plant surfaces (Carlile and Watkinson, 1994). The genus *Candida* includes around 154 species. Among these, only six are most frequently isolated in human infections.

They are playing a significant role in postharvest control of fruit diseases. The yeast *C. oleophila* reduced the incidence of green, blue molds and sour rot of citrus fruit and it was also effective against *Penicillium expansum* and *B. cinerea* on apple (Wisniewski *et al*., 1995). Other reports include *C. sake* against major postharvest pathogens of pome fruits (Usall, 1995), and

C. guilliermondii against *Rhizopus* rot of peach fruits (Lima *et al*., 1998), Controlling postharvest decay by *C. reukaufii* and *C. pulcherrima* on strawberry fruit (Guinebretiere *et al.*, 2000) and by *C. saitoana* on orange, lemon and apple. All of the above reports tell us the potential of yeasts to control fungal post harvest diseases of fruits. All these studies support the findings in our study.

5.4. In vivo Effect of Antagonists against on orange blue mold Development

Potential yeast isolates which show best antagonistic activity against the growth of orange blue mold during in vitro tests were also tested in vivo. Three yeast isolates (**Y_3, Y_{10} and Y_{16}**) were evaluated for their antagonistic effect against the growth of orange blue mold on artificially inoculated orange fruits. Potential yeast isolates which reduced the growth of orange blue mold in in vitro test were also reduced the severity of the disease on artificially inoculated orange fruits.(Tabe-7).However, none of the antagonists were able to completely inhibit lesion development. This is in agreement with previous reports by Koomen and Jeffries (1993).

The presence of zones of inhibition *in vitro* is found to be good predictor of field performance of *Agrobacterium radiobacter* in controlling *Agrobacterium tumefaciens* on peaches (Alconero, 1980). Broadbent et al. (1971) tested 3500 microorganisms for antibiosis *in vitro*. Approximately 40% of these microorganisms inhibited one or more of nine pathogens on agar, and about 4% of these were effective biocontrol agents in soil. The authors noted that, while only some microorganisms that inhibited pathogens on agar also did so in soil, those organisms ineffective on agar were also ineffective in soil. These reports tells us antagonistic organisms which can reduce in vitro can also reduce disease severity in vivo and also in the field the result of this study also agree with above reports.

Table-7: Effect of antagonists on blue mold disease severity of artificially inoculated orange fruits

Code yeasts	Mean ± SD	% disease severity resistant of antagonists
Y16	55.66±5.13[b]	30.90
Y3	62.33±8.14[b]	22.62
Y10	61.00±3.00[b]	24.28
control	80.56±2.89[a]	_

A B

Fig-7 Inhibitory effect of potential yeast isolates against orange blue mold on artificially inoculated orange fruits incubated for 12days at 25^0c. A= orange fruits which were treated with antagonistic yeast isolates. B= orange which was not treated with potential antagonistic yeast isolates.

6. CONCLUSIONS

Postharvest disease of fruits and vegetables is serious problem, especially in developing countries where appropriate preservative methods are not adequately available for more growers. In Ethiopia orange blue mold is one of post harvest disease of orange fruit. The conventional chemical based control strategies of post harvest fruit disease is costly for most growers and has toxicity health risk. In this study the use of selected yeast isolates for controlling of orange blue mold has reveled at promising. Therefore biological control of postharvest fungi disease of fruits including orange blue mold could be potential alternative to chemical methods, and is safe for human health.

7. RECOMMENDATION

Based on the research finding, the following points are recommended:

- In this study, the biological control of orange blue mold disease using yeast has been examined at laboratory level where the exact potential of the antagonistic yeasts on field was not undertaken. Hence, further stud will be required to test the efficiency and effectiveness of the yeasts in antagonizing the pathogenecity of the fungi in the field application.

8. REFERENCE

Alconero, R.(1980). Crown gall of peaches from Maryland, South Carolina, and Tennessee and problems with biological control. *Plant Dis*. **64**: 835-838.

Barkai-Golan, R.(1966). Reinfestation of citrus fruits by pathogenic fungi in the packing house. *Israel. J. Agric. Res*. **16**: 133-138.

Barnett, J.A., Payne, R.W.and Yarrow, D. (2000). **Yeasts: Characteristics and Identification**. Cambridge University Press, UK.

Broadbent, P., Baker, F.K. and Waterworth, Y. (1971). Bacteria and actinomycetes antagonisticto fungal root pathogens in Australian soils. *Aust*. *J*. *Biol*. *Sci*. **24**: 925-940.

Brown, G.E. and Wilson, W.C. (1968). Mode of entry of *Diplodia natalensis* and *Phomopsis citri* in florida oranges. *Phytopathol*. **58**: 736-739.

Brown, G.E. (1975). Factors affecting postharvest development of *Colletotrichum gloeosporioides* in citrus fruits. *Phytopathol*. **65**: 404-409.

Calvente, V., Benuzzi,D. and Tosetti, M.I.(1999). Antagonistic action of siderophoresfrom *Rhodotorula glutinis* upon the postharvest pathogen *Penicillium expansum*. *Inter. Biodeterior. Biodegrad*. **43:** 167-17.

Citrus Commodity Notes, (2005). Developments in international citrus trade in 2004-2005. http://www.fao.org/es/esc/en/20953/20990/highlight_28187en_p.html.

Citrus sinensis information from NPGS/GRIN. Ars-grin.gov. Retrieved on 2011-10-02.

Cook, R.I. and Baker, K.E. (1983). **The National Practice of Biological Control of Plant Pathogens**. American Phytopathological Society, St. Paul.

Dood, J., Bugante, R., Koomen, I., Jeffries, P. and Jaeger,M. (1991).Pre- and Postharvest control of mango anthracnose in the Philippines. *Plant. Patho*.**40**: 576-583.

Ehsani.R et al. "In-situ Measurement of the Actual Detachment Force of Oranges Harvested by a Canopy Shaker Harvesting Machine". *Abstracts for the 2007 Joint Annual Meeting of the Florida State Horticulture Society*. (June, 2007).

Ethiopian Horticulture Development Agency (2003).Post-harvest sector challenges and opportunities in Ethiopia.

Ethiopian Horticulture Development Agency(2008). The status of Post-Harvest and Transport Technologies for Horticulture produces in Ethiopia.

Eyob, S. (1997). Rehabilitation of old citrus orchards. *Rev. Plant Pathol.***76**:741.

FAO (Food and Agriculture Organization of the United Nations). (2004). Production Data Base. FAO/WFP. Rome. http//www.fao.org.

FAO Statistics Top_production_oranges_(2010). Retrieved 26 November 2012.

FAOSTAT". FAO Statistics. Retrieved 2012-09-10.

Fawcett, H.S. and Berger,W.R. (1927). Relation of temperature for the growth of *Penicillium italicum* and *P. digitatum* and to citrus fruit decay produced by these fungi *J. Agric. Res*.

35: 925-931.

Gorinstein, S., Martin-Belloso, O., Park, Y., Haruenkit, R., Lojek, A., Milan, I., Caspi, A., Libman, I., Trakhtenberg, S.(2001). Comparison of some biochemical characteristics of different citrus fruits. *Food Chem.* **74** : 309-315.

Guinebretiere, M., Nguyen, C.,Morrison, N., Reich, M. and Nicot, P.(2000). Isolation and characterization of antagonists for the biocontrol of the postharvest wound pathogen *Botrytis cinerea* on strawberry fruits. *J. Food Prot*. **63**:386-94.

Gutter, Y. (1961). On the pathogencity of *Trichoderma viride* to citrus fruits. *Bul. Res.Council Israel*. **10**: 157-164.

Herath, E., Amano, B., Endale, G., Seyfu G. (1994). Fruit crops improvement research. In: **Horticultural Research and Development in Ethiopia**, pp. 53-62 (Edward, H. and Lemma, D,eds.).EHRA, Addis Ababa, Ethiopia

Hough, J.A., Kellerman, C.R. and Fourie, L.(1980). Pre-harvest spray for brown rot control of citrus. *j.Citr and Subtropic Fruit* . **556**: 10-11.

Ibrahim,S.and Rahma,M.A.(2009).Isolation and identification of fungi associated with date fruits sold at bayero university. *Bjopas*. **2**: 127-130.

Ismail, M. and Zhang, J.(2004). Postharvest citrus diseases and their control. *Outloo Pest.Manag*. **1**: 29-35.

Jeyarajan, R. and Nakkeeran, S. (2000). Exploitation of micro organisms and viruses as biocontrol agents for crop disease management. In: **Biocontrol Potential and its**

Exploitation in Sustainable Agriculture, pp. 9-23 (Updadhyay, R.H., Mukerji,K.G. and Chamola, B.P.,eds.). Kluwer Academic, New York.

Kavanagh, J.A. and Wood, R.K.S.(1967). The role of wounds in the infection of oranges by *Penicillium digitatum* Sacc. *Ann. Appl. Biol*. **60**: 375-383.

Koomen, I. and Jeffries, P. (1993). Effects of antagonistic microorganisms on the postharvest development of *Colletotrichum gloeosporioides* on mango. *Plant Pathol*. **42**: 230-237.

Kreger-Van Rij, N.J.W. (1984).**The Yeast: A Taxonomic Study**. Elsevier Science Publishing Company, New York.

Kurtzman, C.P. and Fell, J.W. (1997). **The Yeasts: A Taxonomic Study.** Amsterdam: Elsevier Science Publishing Company, Amsterdam.

Kurtzman, C.P., Robnett, C.J., Ward, J.M., Bravton, C.,Gorelic, P. and Walsh,T.M.(2005). Multigene phylogenetic analysis of pathogenic *Candida* species in the *Kazachstania* (*Arxiozyma*) *telluris* complex and description of their ascosporic states as *Kazachstania bovina* sp. nov., *K. heterogenica* sp. nov., *K. pintolopesii* sp. nov , and *K. slooffiae* sp. nov. *J. Clin. Microbiol.* **43:**101-111.

Leben, C. (1965). Epiphytic microorganisms in relation to plant disease. *Annu. Rev. Phytopathol.* **3**: 209- 230.

Libman, I., Trakhtenberg, S. (2001). Comparison of some biochemical characteristics of different citrus fruits. *Food Chem*. **74** : 309-315.

Lima, G.,Ippolito,A., Nigro,F. and Salerno,M. (1997). Effectiveness of *Aureobasidium pullulans* and *Candida oleophila* against postharvest strawberry rot. *Postharvest Biol. Technol*.**10:** 169-178.

Lima, G., DeCurtis, F., Castoria, R. and Decicco,V. (1998). Activity of the yeasts*Cryptococcus laurentii* and *Rhodotorula glutinis* against postharvest rots in different fruits. *Biocon. Sci.Technol.* **8**: 257-267.

Lima, G. Arru, S.,Decurtis, F.,Arras, G. (1999). Influence of antagonistic, host fruit and pathogen on the biological control of postharvest fungal diseases by yeasts. *J. Ind. Microbiol. Biotechnol*.**23**: 223–229.

Mc-keen,C.D.,Relly,C.C.and pusey,P.L.(1986).Production and partial characterization of antifungal substances *to Monilinafructicola frobacillus subtilitis.phytopathol*.**76**:136-139.

Mengistu, H. and Amare, A. (1996). **Postharvest Diseases of Food Crops**. Teaching Method Series, Alemaya University.

Moubasher,A. (1993). **Soil Fungi in Qatar and Other Arab Countries**.The center of scientific and applied research and university of Qatar,Doha.

National Academy of Science. (1987). **Postharvest food losses in developing countries.** *U.S National research council*, Washington DC.

Narair, P. (1998). Adialectical perspective agricultural research for sustainable development. C*urr-Sci* .**74**:663-665

Narayanasamy,P.(2005). **Microbial Plant Pathogens and Crop Disease Management.**Science Publishers, Inc., Enfield.

Nautiyal, C.S. (2000). Biocontrol of plant diseases for agricultural sustainability. In: **Biocontrol Potential and its Exploitation in Sustainable Agriculture,** pp. 95-116(Updadhyay, R.H., Mukerji, K.G. and Chamola, B.P.,eds.). Kluwer Academic, New York.

Patiño-vera, M.,Jimenez,B., Baldreras, K., Oritz, M.,Allende, R., Carrillo,A. and Gaindo, E.(2005). Pilotscale production and liquid formulation of *R. minuta,* a potential biocontrol agent of mango anthracnose. *J. app. Mic. Biol.* **99**: 540.

Peng, G., Sutton, J.C. and kevan, P.G. (1992).Effectiveness of honey bees of applying the biocontrol agent Gliocladium roseum to strawberry flowers to Suppress Batrryscinerea. *Can.J. Plant Pathol*. **14**:117-129.

Pitt,I.J.(1979).**The Genus Penicillium and its Teleomorphic States Eupenicillium and Talaromyces**. Academic press Inc.,London.

Pusely, P.L. and Wilson, C.L. (1985).Potentials for biological control of postharvest plant diseases. *A rev*. *Plant Dis.* **69**: 375-378.

Raper,K.B.,Thom,C. and Fennell,D.I. (1968). **Amanual of the Penicillia**.Hafner publishing co.,Inc.,New York.

Raphael,S.P., Francisco,L.S.,Juliana,F.M. and Paula,B.M.(2008). Biological control of *Penicilliu italic, Penicilliu digitatum* and *Penicillium expansum* by the predacious yeast *saccharomycopsis schoeniiemlaranjas*. *Braz.J.Microbiol.* **39**:1517-8382.

Roberts, R.G. (1990). Postharvest biological control of gray mold of apple by *Cryptococcus laurentii*. *Phytopathol*. **80**: 526-529.

Roth, G. (1967). Citrus fruit decay in South Africa caused by *Penicillium digitatum* Sacc. *Phytopathol. Z*. **58**: 383-396.

Sissay Bekele (2007). **Identification of Citrus(citrus sinensis) Postharvest Pathogens from Ethiopian and their Control**. faculty of Natural and Agricultural sciences, Pretoria.

Smilanick, J.L.(1994). Strategy for isolation and testing of biocontrol agents. In: **Biological Control of Postharvest Diseases: Theory and Practice,** pp.25-42(Wilson,L.C. and Wisniewski, E.M.,eds.).CRC Press, Florida.

Smoot, J.J., Houck, L.G. and Johnson, H.B. (1983). Market diseases of citrus and other subtropical fruits. ARS, Washington DC.

Spalding, D.H. (1982).Resistance of mango pathogens to fungicides used to control postharvest diseases. *Plant Dis.***66**:1185-1186.

Stange,R.R. and Eckert, J.W. (1994). Influence of postharvest handling and surfactants on control of green mold of lemons by curing. *Phytopathology*. **84**: 612-616.

Thompson, A.K. (1987) .The development and adaptation of methods for control of anthracnose. In: **Mangoes Review,** pp. 29-38 (Prinsley, R.T. and Tucker, G.,eds.). Common Wealth science council, London.

USDA Foreign Agricultural Service. "USDA – U.S and the World Situation: Citrus".

Usall, J., Teixido,N., Fons,E. and Vinas,I. (2000). Biological control of blue mould on apple by a strain of *Candida sake* under several controlled atmosphere conditions. *Int. J. Food Microbiol.* **58**: 83-92.

Wijedaso,M.H.and Lianapathirana,L.V.(2012).Evaluation of an alternative slid culture technique for the morphological identification of fungi species.*Sir.J.infec.dise*.**2**:47-52.

Wilson, C. L. and Chalutz, E. (1989). Postharvest biological control of Penicillium rots of citrus with antagonistic yeast and bacteria. *Sci. Hortic*.**40**:105-112.

Wisniewski, M. E., Droby, S., Chalutz, E. and Eilam, Y. (1995). Effect of Ca21 and Mg21 on *Botrytis cinerea* and *Penicillium expansum* in vitro and on the biocontrol activity of *Candida oleophila*. *Plant Pathol*. **44**: 1016-1024.