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Antifungal study of garlic (Allium sativum) extract on the Fusarium spp. isolated from muskmelon fruit rot / Gan Chie Giap.

Lihat sebelah

ANTIFUNGAL STUDY OF GARLIC (*Allium sativum*) EXTRACT ON THE *Fusarium spp.* ISOLATED FROM MUSKMELON FRUIT ROT

By
Gan Chie Giap

Research Report submitted in partial fulfillment of
the requirement for the degree of
Bachelor of Science in Agrotechnology (Post Harvest Technology)

DEPARTMENT OF AGROTECHNOLOGY
FACULTY OF AGROTECHNOLOGY AND FOOD SCIENCE
UNIVERSITI MALAYSIA TERENGGANU
2010

ENDORSEMENT

The project report entitled **Antifungal Study of Garlic (*Allium sativum*) Extract on the *Fusarium spp.* Isolated from Muskmelon with Fruit Rot** by **Gan Chie Giap,** Matric Number **UK 15866** has been reviewed and corrections have been made according to the recommendations by examiners. This report is submitted to the Department of Agrotechnology in partial fulfillment of the requirement of degree of Science in Agrotechnology (Post Harvest Technology) Faculty of Agrotechnology and Food Science, Universiti Malaysia Terengganu.

.....

(MISS SITI NORDAHLIAWATE MOHAMED SIDIQUE)
Main supervisor

SITI NORDAHLIAWATE MOHAMED SIDIQUE
Lecturer
Department of Agrotechnology
Faculty of Agrotechnology and Food Science
Universiti Malaysia Terengganu
21030 Mengabang Telipot

Date: 25/4/10

.....

(MISS ROSHITA IBRAHIM)

Co-supervisor

ROSHITA IBRAHIM
Pensyarah
Jabatan Agroteknologi
Fakulti Agroteknologi dan Sains Makanan
Universiti Malaysia Terengganu

Date: 25/4/10

DECLARATION

I hereby declare that the work in this thesis is my own except for quotations and summaries which have been duly acknowledged.

Signature	: .....
Name	: Gan Chie Giap
Matric Number	: UK 15866
Date	: 25 April 2010

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ABSTRACT

Muskmelon is the common name for botanical varieties of *Cucumis melo L.* The botanical group includes cantaloupe, honeydew, and casaba. Muskmelon fruit rot has been detected in almost plantation area and *Fusarium* species had been identified involved in one of the most important postharvest diseases of muskmelon. This study was employed to determine the effect garlic extraction on causal organisms and the Minimal Inhibitory Concentration (MIC) of *Fusarium Solani* and *Fusarium Oxysporum.* Three types of in-vitro techniques were tested (Paper disc diffusion, well-agar technique and spore suspension techniques). Results showed that 5mg/ml of garlic extract was proven can inhibit both of the fungi. The MIC of *F. Solani* and *F. oxysporum* were 4.75 mg/ml and 4.5 mg/ml respectively. As conclusion, garlic has a potential as natural product to inhibit the *Fusarium* species isolated from muskmelon with fruit rot disease.

ABSTRAK

Cucumis melo L. atau dikenali dengan nama botanical iaitu tembikai terdiri daripada tembikai susu, 'cantaloupe dan 'casaba'. Penyakit reput buah telah dikesan pada setiap kawasan penanaman buah tembikai. *Fusarium* spesies telah dikenalpasti punca kepada penyakit lepastuai bagi buah tembikai. Kajian ini dijalankan untuk mengkaji kesan kepekatan ekstrak bawang putih terhadap perencatan *Fusarium* spesies dan menentukan kepekatan perencatan minimum (MIC) bagi *Fusarium solani* dan *Fusarium oxysporum*. Tiga jenis teknik in-vitro telah digunakan (Teknik sebaran kertas cakera, teknik 'agar-well' dan teknik ampaian inokulum). Keputusan menunjukkan ekstrak bawang putih pada kepekatan 5mg/ml dapat merencat kedua-dua kulat tersebut. Penentuan kepekatan perencatan minimum bagi *Fusarium solani* dan *F. oxysporum* adalah 4.75 mg/ml dan 4.5 mg/ml masing-masing. Sebagai kesimpulannya, bawang putih mempunyai potensi sebagai produk semulajadi untuk merencatkan pereputan pada buah tembikai disebabkan oleh *Fusarium*.

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LIST OF ABBREVIATIONS

°C	-	Degree centigrade
%	-	Percentage
rpm	-	Rotary per minute
DMSO	-	Dimethyl sulphoxide
MIC	-	Minimal Inhibitory Concentration
≤	-	Same with or less than
≥	-	Same with or more than
CLA	-	Carnation Leaf Agar
WA	-	Water Agar
cm	-	Centimeter
mm	-	Milimeter
g	-	Gram
mg	-	Miligram
µl	-	Microliter
ml	-	Mililiter
PDA	-	Potato Dextrose Agar
PDB	-	Potato Dextrose Broth
Spp.	-	Species

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CHAPTER 1

INTRODUCTION

1.1 Background of Study

Plant diseases are introduced to human beings nearly 4000 years ago since they cause damage to several plants upon which human subsist on for their food, clothing, housing material and more. In recent years, intensive cropping for agriculture development has increased the disease incidences consider. Many plant pathogens, such as fungi, bacteria, nematodes, viruses and mycoplasma create serious problems to crop plants at various stages of growth from sowing to postharvest resulting in major losses (Chattopadhyay and Mustafee, 2007).

Commonly, the harvested fruits or vegetables which ready to be marketed need a certain storage period causes by overstocked and low demand in market. Besides that, fresh fruits and vegetables destined for export also require long periods of storage and shipment prior to arrival. In the course of storage and shipment part of the fresh produce, which is rich in moisture and nutrients and therefore may serve as a suitable substrate for the development of microorganisms, may rot and become unfit for sale (Barkai-Golan, 2001).

The growing awareness of consumers concerning on the relation between food and health is revolutioning in the food industry. New technique such as high pressure, nanotechnology and irradiation, are increasingly needed to maximize the nutritional

properties contribute to improving health. Most advanced technologies also access in handling fresh fruits and vegetables, such as computerized sorting and grading, improved packing materials and methods, and advanced regular or modified atmosphere storage, still losses caused by post harvest diseases remain substantial. In fact, international agencies that monitor world food resources have acknowledged that one of the most feasible options for meeting future needs is the reduction of postharvest losses (Kelman, 1984). The "elimination" of additives used in a wide variety of foods is demanded, while "natural" additives are seen as a benefit for both quality and safety (Martos *et al*. 2008).

1.2 Problem Statement

The widespread use of pesticides has significant drawbacks including increased cost, handling hazards, concern about pesticides residues on food, and threat to human health and environment (Paster & Bullerman, 1988). Fungicides are primary means of controlling postharvest disease. Their world-wide use is variable, comprising 26% of the plant protection market in Europe and Asia and 6% in the US (Jutsum, 1988). About 23 million kg of fungicides are applied to fruit and vegetables annually, and it is generally accepted that production and marketing of these perishable products would not be possible without their use (Tripathi & Dubey, 2004).

However, as harvested fruit and vegetable are commonly treated with fungicides to retard postharvest disease, unfortunately their usage has been restricted due to greater expose to human and can cause carcinogenicity, teratogenicity, high and acute residual toxicity, long degradation period, environmental pollution and their

effects on food and other side-effects to humans (Lingk, 1991; Unnikrishnan and Nath, 2002). As well, phytotoxic and off-odour effects of some prevalent fungicides have limited their use. One problem with these synthetic chemicals is that as their potency has been enhanced, so has been their side-effects, and also their cost (Tyler, 1992; Castro *et al.*, 1999; Falandysz, 2000; Kast-Hutcheson *et al.*, 2001; Sorour and Larink, 2001). The side-effects of synthetic fungicides means that alternative strategies need to be developed for reducing losses due to postharvest decay that are perceived as safe by the public and pose negligible risk to human health and environment (Wilson *et al.*, 1999).

1.3 Significant of Study

According to Tegegne *et al.* (2008), extensive investigations into possible exploitation of plant compounds as natural environment have been undertaken over the past two decades cause in an attempt to find out the way for reducing the use of synthetic pesticides. One such alternative in the use of natural plant protectants with pesticidal activity, as well as they tend to have low mammalian toxicity, less environmental effects and wide public acceptance (Tzortzakis and Economokis, 2007).

Considerable attention has also been given to the potential of biological control of postharvest diseases of fruit and vegetables as a viable alternative to the use of present day synthetic fungicides (Wilson and Wisniewski, 1989; Wilson *et al.*, 1999; Pang *et al.*, 2002). These drawbacks in alternative methods have increased interest in developing further alternative control methods, particularly those which are environmentally sound and biodegradable. Thus, replacement of synthetic fungicides

by natural products (particularly of plant origin), which are non-toxic and specific in their action, is gaining considerable attention. The fruits decay caused by plant pathogenic fungi can be managed with exploitation of natural products such as flavour compounds (e.g. acetaldehyde, benzaldehyde, hexanal, acetic acid, jasmonates, glucosinolates, propolis fusapyrone and deoxyfusapyrone, chitosan, essential oils, active principles of some plants extracts (Tripathi and Dubey, 2004).

The preservative nature of some plant extracts has been known for centuries and there has been renewed interest in the antimicrobial properties of extracts from aromatic plants. Some plants extracted in different organic solvents have shown inhibitory action against different storage fungi (Singh *et al.*, 1993; Mohamed *et al.*, 1994; Hiremath *et al.*, 1996; Kapoor, 1997; Radha *et al.*, 1999; Rana *et al.*, 1999).

1.4 Objectives

My study objectives are:

1. To examine the effect of garlic extraction on muskmelon fruit rot causal organisms
2. To determine the minimal inhibitory concentration of the effectiveness of extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Postharvest Diseases of Muskmelon (Fruit Rot)

Muskmelon is the common name for botanical varieties of *Cucumis melo L.* This botanical group includes cantaloupe, honeydew, and casaba. Cantaloupe fruit (*C. melo var. reticulatus Naud.*) are usually characterized by netted surfaces with shallow vein tracts and the flesh is salmon-coloured. Honeydew and Casaba fruit (*C. melo var. inodorus Naud.*) typically have unnetted surfaces. This group also includes Persian and Crenshaw melons. Honeydew fruit have smooth, white skin with green flesh, while Casaba fruit have yellow, green or white skin, which are usually rough and wrinkled (Agblor and Waterer, 2001).

Cantaloupes harvested at full maturity are high in sugars, and have good flavour and aroma. At full slip an abscission layer forms allowing the melon to separate from the vine, leaving no stem tissue attached to the fruit. Cantaloupes harvested prior to full slip are not as high in sugars and flavour but have longer storage potential. To ensure a shelf life of up 2 weeks, a compromise has to be made between harvest dates. Muskmelon (*Cucumis melo* var. *cantaloupensis* Naud.) is a very commercial horticulture crop in domestic consumption in Malaysia and also significant quantities for export. Unfortunately, the main factor limiting export is the short shelf-life and development of associated post harvest decays (Bruton, 1995).

Hard-ripe muskmelon has a normal postharvest shelf-life of about 14 days under proper storage conditions (Lester, 1988; Lester and Stein, 1993). However, fruit firmness declines by 16% after 10 days storage and 37% after 20 days storage (Lester and Dunlap, 1985; Lester, 1988). As muskmelon fruit soften, postharvest decays increase significantly (Bruton, 1995). *Fusarium* species had been identified involved in one of the most important postharvest diseases of netted muskmelon (Bruton, 1995). Any diseased, overripe or damaged fruit should be culled, otherwise the spoiled fruit will contaminate healthy fruit.

2.2 The Causal Organisms

Fusarium fruit rot is one of the most common preharvest and postharvest soil-borne diseases of muskmelons and planting resistant cultivars is the best way to protect the crop. Symptoms of Fusarium fruit rot vary depending on the *Fusarium* species and the host. The fungi are including *F. graminum Coda*, *F. graminearum Schwabe, F. acuminatum Ellis* & Everh. sensu Gordon, *F. avenaceum* (Fr.:Fr.) Sacc., *F. culmorum* (W. G. Sm.) Sacc., *F. moniliforme* J. Sheld., *F. semitectum* Berk. & Ravenel, *F. equiseti* (Corda) Sacc. sensu Gordon, *F. scirpi* Lambotte & Fautrey, *F. solani* (Mart.) Appel & Wollenweb. emend. W. C. Snyder & H. N. Hans., and *F. oxysporum* Schlechtend.:Fr. f. sp. Melonis (Bruton and Duthie, 2000).

This fungus is soil-borne but it can also be carried by the seed. It can survive up to three years in the soil or the seed. Spores of the fungus are produced on the infected plant material and can be transported by rain splash, running water, cultivation, and any other practice which transports soil around the field. The

heaviest concentrations tend to be where infected plants were previously grown (Watt, 2004).

Fruit may be infected and the infection usually begins on the soil side. Fruit infections appear circular although multiple infections may merge and obscure concentric patterns. Fusarium fruit rot is usually firm and dry although secondary organisms may invade and cause a wet rot (Watt, 2004). In the case of *Fusarium oxysporum* f. sp. melonis infection of melon, *Fusarium* is rare in directly penetrates the epidermis but, it invades the fruit through the stem end. As postharvest breakdown proceeds, other secondary fungi like the dark mycelial growth of Alternaria rot (*Alternaria alternata*) may also be found (Zitter, 1998).

All cucurbits families are susceptible to one or more species of *Fusarium.* The fungus may penetrate directly under moist or wet conditions. Wounds will facilitate fungal entry. Most infections of fruit occur in the region that is in contact with the soil. Although uncommon in watermelon fruit, *Fusarium* spp. can infect at the stem end and, less frequently, at the blossom end and belly. Fusarium rot is a fairly common fruit rot of pumpkin and squash, as both a preharvest and postharvest decay (Bruton and Duthie, 2000). Most infections of cucurbits by *Fusarium* spp. occur in the field (preharvest) and, to a lesser extent, during harvesting and handling. Preharvest fungicide application has been somewhat ineffective, because of difficulty in obtaining sufficient coverage of the fruit. Postharvest control of *Fusarium* rot of melon has also been erratic (Bruton and Duthie, 2000). The diseases are responsible for the greatest losses in yield and also cause severe postharvest losses (Zitter, 1998).

2.2.1 *Fusarium solani & Fusarium oxysporum*

The genus of *Fusarium* was first introduced by link in 1809 and now it had been reported as plant-pathogenic fungi. The members of this genus can incite directly diseases in plants, human and domesticated animal (Boon-pasart *et al.*, 2002; Goldschmiedet *et al.*, 1993; Krcmery *et al.*, 1997; Martino *et al.*, 1994; Rabodonorina *et al.*, 1994). In 1960s, *F. oxysporum* had been reported caused panama wilt on banana industry and can gave a several major impact in the industry (Ploetz, 1990).

First reported in California, United States in 1983 when specificity to cucurbits was demonstrated, the pathogen was renamed *Fusarium solani* f. sp. *cucurbitae*. Subsequently, in the late 1950s, Tousson and Snyder (1961) isolated a second cucurbit pathogen at low frequencies that affected only the fruit, which they named race 2 of *F. solani* f. sp. *Cucurbita* (Bruton, 1995).

Fusarium solani (Mart.) is one of the most important *Fusarium* species involved in both preharvest and postharvest decay of cucurbit fruit. *F*usarium *solani* can cause severe muskmelon (*Cucumis melo* L. var. *cantalupensis* Naud.) fruit decay by natural infection or after artificial inoculation. The taxonomic position of strains identified as *F. solani* is currently somewhat problematic (Zhang *et al.*, 1998).

Other than that, *F. oxysporum f. sp. melonis* is also a major postharvest disease problem in muskmelon (*Cucumis melo L)* resulting in significant postharvest losses (Huang *et al.* 2000). The infection can enter the flower at anthesis and remain latent in the field throughout the growing period, with symptoms appearing as the fruit ripens or during postharvest storage (McConchie *et al.*, 2007).

2.3 Garlic as Antifungal Agent

Garlic (*Allium sativum*) is a member of the Lily family, which contains over 6,000 species including well-known edible plants such as onion, chives, leek, and shallot (Wilder, 2008). The ancient Egyptians, Greeks, Chinese, Indians and Romans all advocated the therapeutic value of garlic in the treatment of ailments ranging from eye disorders, sore throats, headaches, bites, worms and tumours (Wilder, 2008).

Scientific research on these plants started in the second half of the 19th century with Pasteur, who evidenced the antibacterial properties of garlic (Najjaa *et al.*, 2007). Recent investigations have demonstrated an inhibitor effect by aqueous extracts on numerous bacterial and fungal species (Sivam, *et al,* 1997; Phay *et al.*, 1999; Hsieh *et al*, 2001; Ward, *et al.* 2002). During the last 50 years, protection of food from spoilers and pathogens aroused great interest and was achieved by various physical and chemical methods. Among these numerous and abundant naturally occurring compounds, essential oil extracts have been considered as natural preservatives or food additives, and can be used as additional methods of controlling pathogens (Naidu, 2000).

Several Allium species such as *Allium sativum* and *Allium cepa* have been shown in previous studies to exhibit various activities. For example, a wide array of therapeutic effects of garlic has attracted particular attention of modern medicines because of its widespread use as antiatherosclerotic, antidiabetic, antihypertensive, antimicrobial, anticancerous, antioxidant, antifungal and antiviral. Allium species, namely, onion, garlic, leek, and chivecontain a variety of secondary sulphur compounds. Sulphur-carrying flavour compounds are responsible for the characteristic smell and taste, the source of major active compounds which are the

best known properties in Allium plants (Najjaa *et al.,* 2007). Garlic has been shown to inhibit the growth of a variety of microorganisms, not only bacteria but also fungi and viruses. The antimicrobial activity of garlic is believed to be due to the effect of allicin, the main ingredient in garlic, generated by the phosphopyridoxal enzyme allinase (Yoshida *et al.,* 1987).

Alliums were revered to possess antibacterial and antifungal activities, and contain the powerful sulfur and other numerous phenolic compounds which arouse great interest (Rivlin, 2001; Griffiths, *et al.,* 2002). Onions and garlic are composed mainly of water (85–90 g/100 g and 60–70 g/100 g fresh weight, respectively) and the most significant components, medicinally, are the organosulfur-containing compounds. However, garlic contains nearly three times as much sulfur-containing compounds as onions (11–35 mg/100 g fresh weight) (Lawson, 1996). The mature, intact Alliums contain mainly cysteine sulfoxides, and when tissues are chopped, the enzyme allinase is released, converting the cysteine sulfoxides into the thiosulfinates. These compounds are reactive, volatile, odor producing and lachrymatory (Block, *et al.,* 1992). In addition to their nutritional effects, the antibacterial activities against a variety of Gram-negative and Gram-positive were continued to be extensively investigated (Whitemore and Naidu, 2000).

CHAPTER 3

MATERIALS AND METHODS

3.1 Fungal Strains

The Fusarium species were obtained from Postharvest Laboratory fungi collection and maintained on Potato Dextrose Agar (PDA). The morphological identification was based on Leslie and Summerell (2006) after those fungi were growth on carnation leaves (CLA). Two species of *Fusarium*; *Fusarium solani* (D24) and *Fusarium oxysporum* (T08) were used in this study. The *Fusarium* sp. is the causal organism for fruit rot diseases.

Both fungi were single-spored to get the pure culture. Pure culture is one of the essential method for identification of microorganisms. A spore suspension was made by transferring the culture by taking a small portion of mycelia into 10 ml of sterile distilled water. The suspended spore solution was than streaked to the water agar (WA) by using culture loop. As streak progress, the spore become separated and finally the individual colonies appear. A single germinated conidium was removed by using transfer into another PDA and the pure culture of *Fusarium* was obtained that were used throughout this study.

3.2 Garlic Extraction

3.2.1 Sample Preparation

The peeled fresh garlic was dried into the oven at 60°C for two days before ground into fine powder using stainless-steel grinder and stored at room temperature.

3.2.2 Extraction of Plant Material

The 10 g of garlic powder was shaked vigorously with 50 ml of 100% Ethyl-Alcohol for 24 hour by using orbital shaker with the speed 200 rpm. After centrifugation process, the filtrates were collected and evaporated to dryness in a rotary evaporator.

3.2.3 Extraction dilution

Serial dilution technique was used to prepare the garlic extract solution before performing the in vitro test. The garlic was dissolved in sterilized distilled water to desired concentration and Dimethylsulphoxide (DMSO) was added.

3.3 In-vitro Techniques

Three in-vitro techniques were employed for determination of antifungal activities; paper disc diffusion technique, spore suspension technique and agar-well diffusion technique for antifungal activities of garlic extraction. The procedures were

performed in the laminar flow cabinet, the Potato Dextrose Agar (PDA) medium was poured into sterile plastic Petri dishes, and waited until solidified before run the techniques. After incubation for 24 hour at 37°C, all plates for each technique were examined for any zones of growth inhibition, and the diameter zones were measured in millimeters (ml). Sterilized distilled water was used as a control.

3.3.1 Paper Disc Diffusion Technique

In paper disc diffusion method, the garlic extract solution tested were 0.02 mg/ml, 0.2 mg/ml and 2 mg/ml. Paper disc (5 mm diameter) were impregnated with 15 µl of extract and placed on the inoculated plates (Figure 1). The inoculums were removed by using a needle and placed in the centre of the agar medium.

3.3.2 Agar-well Diffusion Technique

In agar-well diffusion method, all extracts were weight and dissolved in sterilized distilled water to three concentrations; 3 mg/ml, 4 mg/ml and 5mg/ml. The wells were prepared by making a hole on the agar by using cork borer and 100µl of extract solution was delivered into them. The measurement of fungi as shown below:

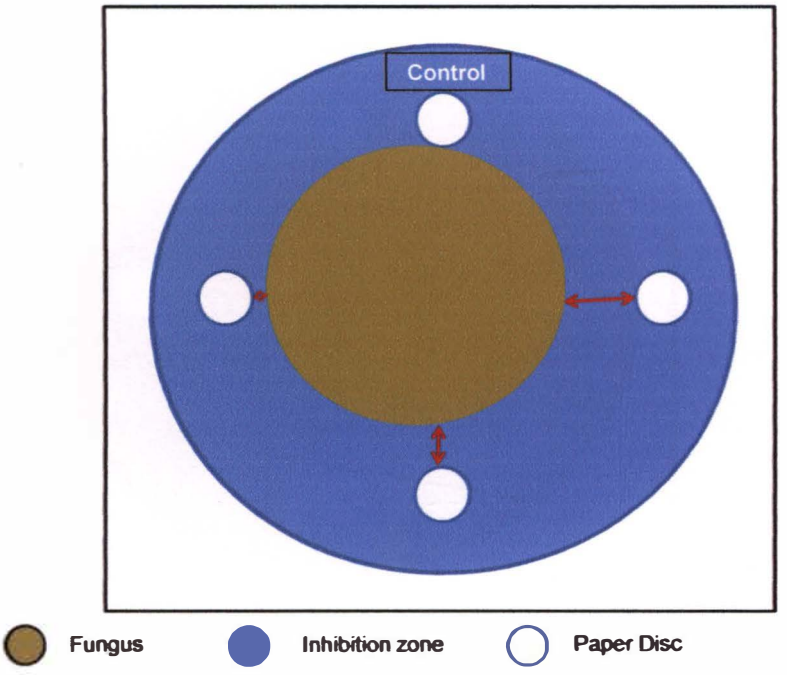


Figure 3.1: Measurement length of mycelia growth by using paper disc diffusion and agar-well diffusion techniques (arrow).

3.3.3 Spore Suspension Technique

The preparation spore suspension using haemocytometer and standardized in 1 x 10^3 conidial/ml was transferred into the PDA and the mixture was shaken gently. The mixture was poured into plate and rotating the plate to create a uniform mixture of spore suspension and left to solidify at room temperature. Three concentrations tested were 3 mg/ml, 4 mg/ml and 5 mg/ml. Paper disc (5 mm) were impregnated with 15µl placed on the surface of solidified agar inoculated with *Fusarium*.

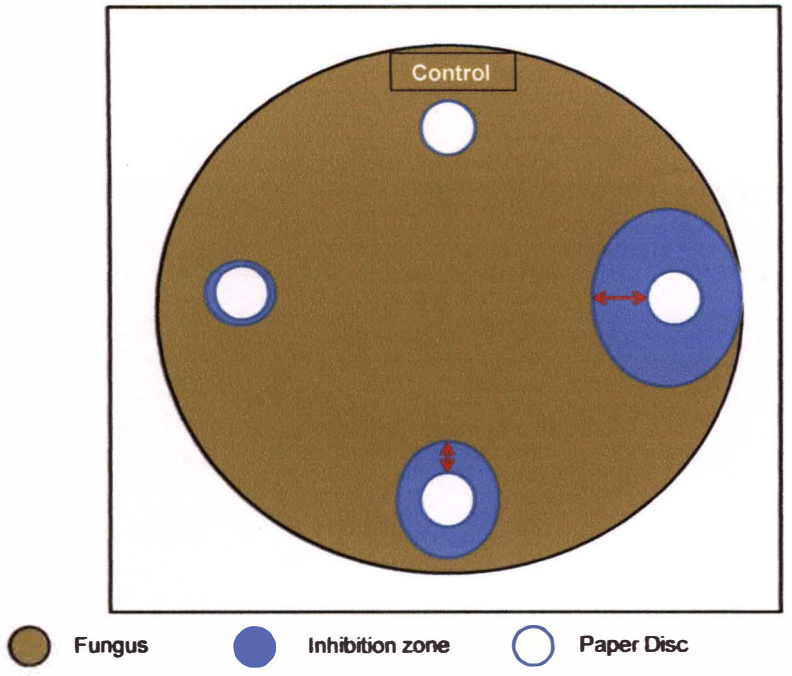


Figure 3.2: Measurement of inhibition zone of spore germination by using spore suspension technique (arrow)

3.4 Minimum Inhibition Concentration (MIC) determination

MIC was defined as the lowest concentration of extract that can inhibit growth of the microorganism (Cellini, *et al.*, 1996). MIC of the samples can be determined by the broth dilution method using different concentration. The fungal cultures were prepared in potato dextrose broth, incubated at 25°C for 48 hour and the concentration of fungal must be standardized (Lee *et al.*, 2006).

The tests were performed in Potato Dextrose Broth (PDB) and fungi strains were standardized in 1 x 10^3 of density which confirmed by viable counts using haemocytometer. The dilution ranging from 4.0 $mgml^{-1}$ to 10.0 $mgml^{-1}$ of the garlic extraction and were prepared in bijou bottle. The tests were performed in triplicate to

confirm the values. Appropriate volume of dilution of broth, extract and fungal added into bijou bottle were as below:

Table 3.1: The volume of extract, broth and *Fusarium (*Broth dilution method)

Bijou bottle	Concentration (mg/ml)	Volume of Extract (µl)	Volume of Broth (µl)	Volume of fungi (µl)
1	10.0	200	1400	400
2	5.00	100	1500	400
3	4.75	95	1505	400
4	4.50	90	1510	400
5	4.25	85	1515	400
6	4.00	80	1520	400
7	Broth	None	2000	None
8	Broth + Fungi	None	1600	400
9	Broth + Garlic Extract	200	1800	None

CHAPTER 4

RESULTS & DISCUSSIONS

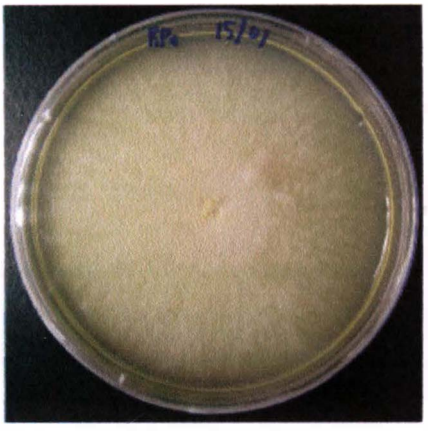
4.1 Morphological Characteristics of *Fusarium solani*

4.1.1 Macroscopic Characteristics

On Potato Dextrose Agar (PDA), cultures of *F. solani* usually are white to cream with sparse mycelium (Figure 4.1A). The colony diameters on PDA are 2.1 until 2.9 cm at 25°C after 3 days cultivations. Many of the isolates do not produce pigments in the agar although some violet or brown pigment may be observed.

4.1.2 Microscopic Characteristics

On Carnation Leaves Agar (CLA), macroconidia were found abundantly in cream and relatively wide, straight, to slightly curved, 3 to 5 septate and also thick walled with short, blunt and sometimes hooked apical cell and notched base to basal call (Figure 4.1B). The false heads relatively long monophialides with oval, ellipsoidal or reniform, 0- or 1- septate microconodia. Chlamydospores often are produced abundantly in pairs in hyphae in the agar.



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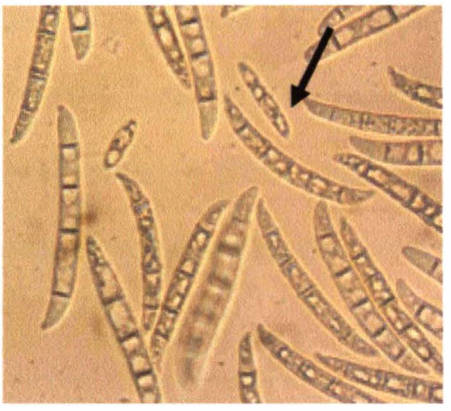


B

Figure 4.1: *Fusarium solani* characteristics: (A) morphological appearance on PDA and (B) the macroconidia (arrow)

4.2 Morphological Characteristics of *Fusarium oxysorum*

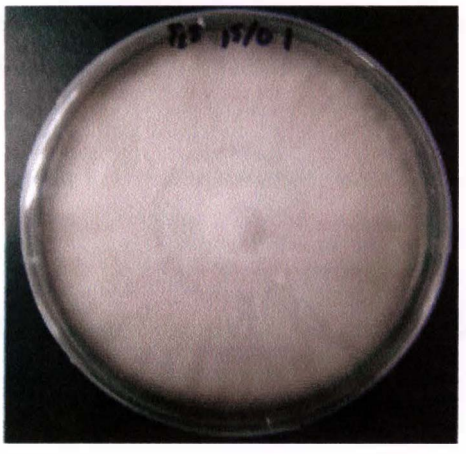
4.2.1 Macroscopic Characteristics

On PDA, colony morphology varies widely. Mycelia may be floccose, sparse or abundant and range in color from white to pale violet. *Fusarium oxysporum* usually produces a pale to dark violet or dark magenta pigment in the agar but some isolates produce no pigment at all (Figure 4.2A).

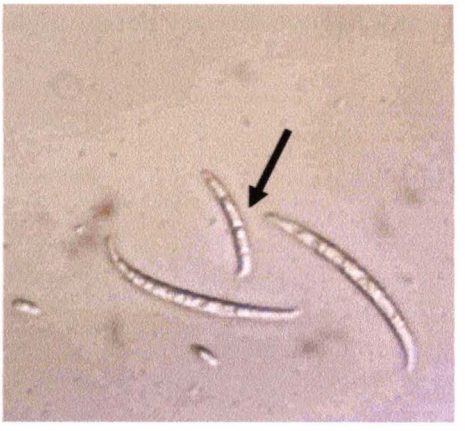
4.2.2 Microscopic characteristics

Macroconidia are formed in pale orange, usually abundant, sporodochia. The marcoconidia are short to medium in length, falcate to almost straight, thin walled and usually 3-septate. The apical cell is short and is slightly hooked in some isolates. The

basal cell is notched or foot-shaped (Figure 4.2B). Macroconidia are formed from monophialides on branched conidiophores in sporodochia and to a lesser extent from monophialides on hyphae. Microconidia usually are 0-septate, may be oval, elliptical or reniform (kidney-shaped), and are formed abundantly in false heads on short monophialides. Chlamydospores are formed abundantly in hyphae on the agar surface.



A



B

Figure 4.2: *Fusarium oxysporum* characteristics: (A) morphological appearance on PDA and (B) the macroconidia (arrow)

4.3 Garlic Extraction Process

The garlic which had been diluted with ethanol by ratio 1:5 for overnight was spin by using rotary evaporator. Only crude form remained after the process fresh garlic 1.3 kilogram was used and only 41 gram was obtained. Therefore, from fresh sample, the recovery of garlic extraction was 3.2%.

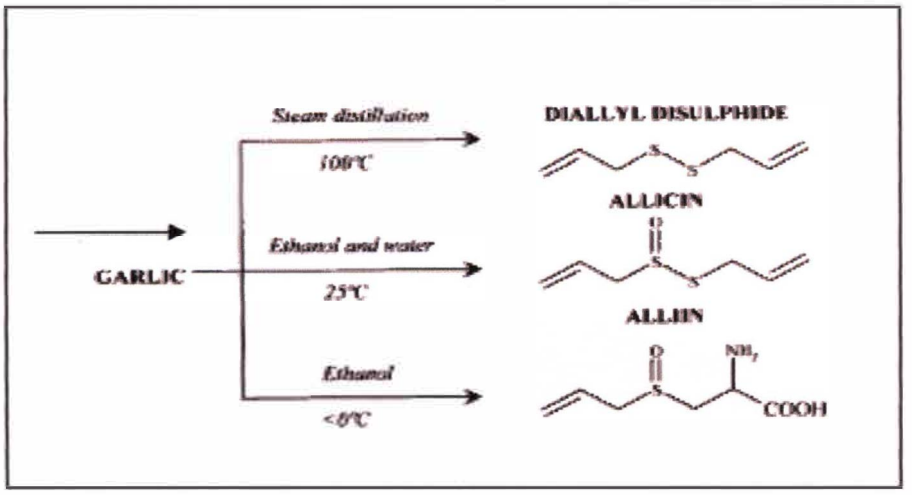


Figure 4.3: Sulfur compounds extracted from garlic under various conditions; garlic diluted by ethanol (arrow) (Harris *et al.*, 2001)

4.4 In vitro Antifungal Activity of Garlic Extract

4.4.1 Paper Disc diffusion technique

Table 4.1: The length of fungi growth (mm) on different garlic extract concentrations

Fungi	Day	Concentrations			
		Control	0.02 mg/ml	0.2 mg/ml	2 mg/ml
F. solani	2	9.0	8.3	8.3	6.0
	4	17.0	17.3	17.7	16.3
	6	27.3	28.0	27.0	26.3
F. oxysporum	2	9.3	9.7	10.0	10.0
	4	20.0	20.0	19.3	18.7
	6	31.0	30.7	29.0	27.3

Garlic extraction exhibited different inhibition level against *F. solani* and *F. oxysporum* as shown in Table 4.1. In the dose response study, the growth of fungal decreased with increasing of concentrations of extracts. The development of fungi at

low concentrations (0.02 mg/ml and 0.2 mg/ml) showed a very low inhibition. After day 6, the effective concentrations were 2 mg/ml for both of *Fusarium* species (Table 4.1 and Figure 4.4).

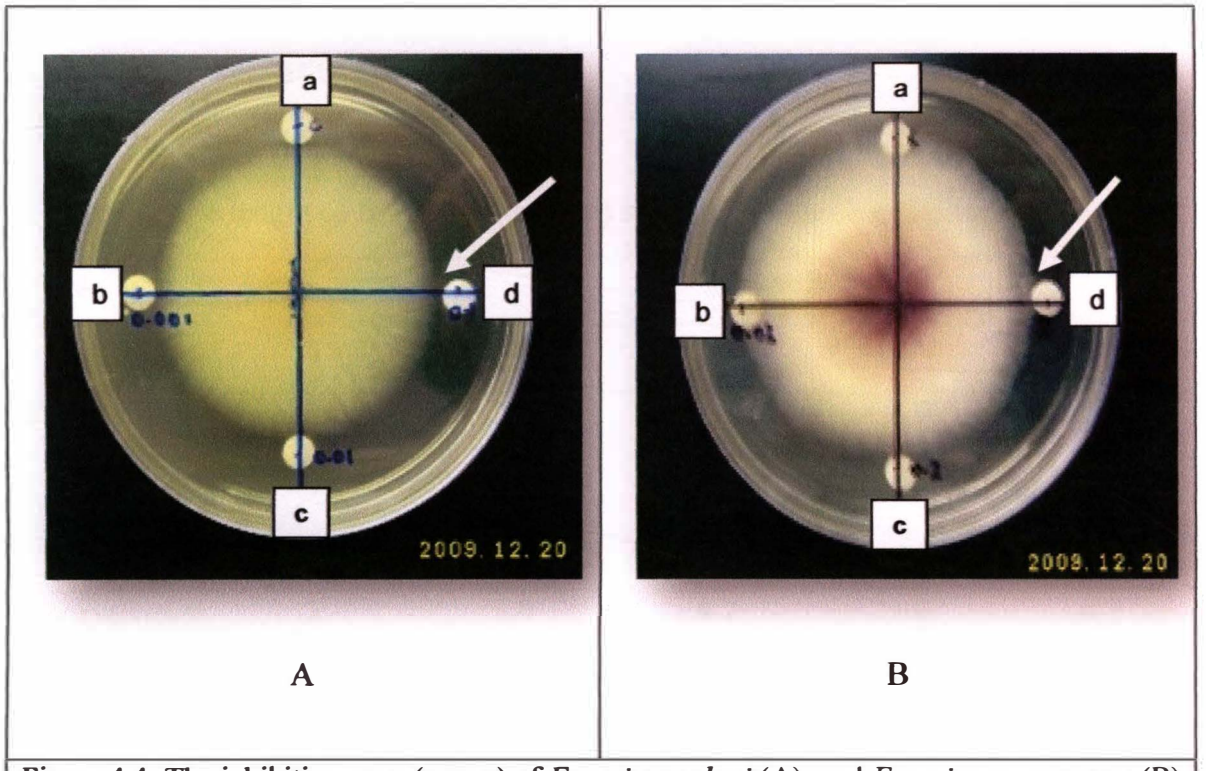


Figure 4.4: The inhibition zone (arrow) of *Fusarium solani* (A) and *Fusarium oxysporum* (B) on day 6.
*a: control, b: 0.02 mg/ml, c: 0.2 mg/ml and d: 2 mg/ml

This technique used to determine the inhibitory properties of the garlic extraction which involved soaking disc with extracts and placing them on the surface of the PDA. There were several problem occurs in this method include their inadequate volume of fungal spores, non uniform of fungal growth, less effectiveness of paper disc and usage of extract.

The fungal inoculated in agar by using a loop or needle gave an accurate volume of spores. The non uniform of fungal growth in the plate also can affect the accuracy of measurement or observation to be done. The paper disc can only absorbed a small amount of extraction and easily evaporated. This will allowed the

fungal to growth faster and fill up the whole plate. Thus, the poor extract retention by the paper disc and losses of active compound of extract properties can let the *Fusarium* species to grow without any inhibitory and once the mycelium reached the paper disc, there was no more extraction left.

4.4.2 Agar-well Diffusion Technique

Table 4.2: The length of fungi growth (mm) by different garlic extract concentrations

Fungi	Day	Concentration			
		Control	3 mg/ml	4 mg/ml	5 mg/ml
F. solani	2	2.7	2.3	1.7	2.0
	4	11.3	11.0	10.7	9.7
	6	23.7	23.3	22.7	22.0
F. oxysporum	2	5.0	4.7	5.0	4.7
	4	14.3	14.0	13.0	10.3
	6	23.3	22.7	22.3	21.0

The well disc diffusion method had been done purposely to improve the effectiveness of paper disc diffusion technique. The agar-well diffusion technique is much higher (3, 4 and 5 mg/ml) than paper disc diffusion technique (0.02, 0.2 and 2 mg/ml) to exhibited against *F. solani* and *F. oxysporum* as shown in (Table 4.2). The differences can be observed between the two methods. High antifungal activity when it showed more inhibition zone for both of *Fusarium* species (Figure 4.5).

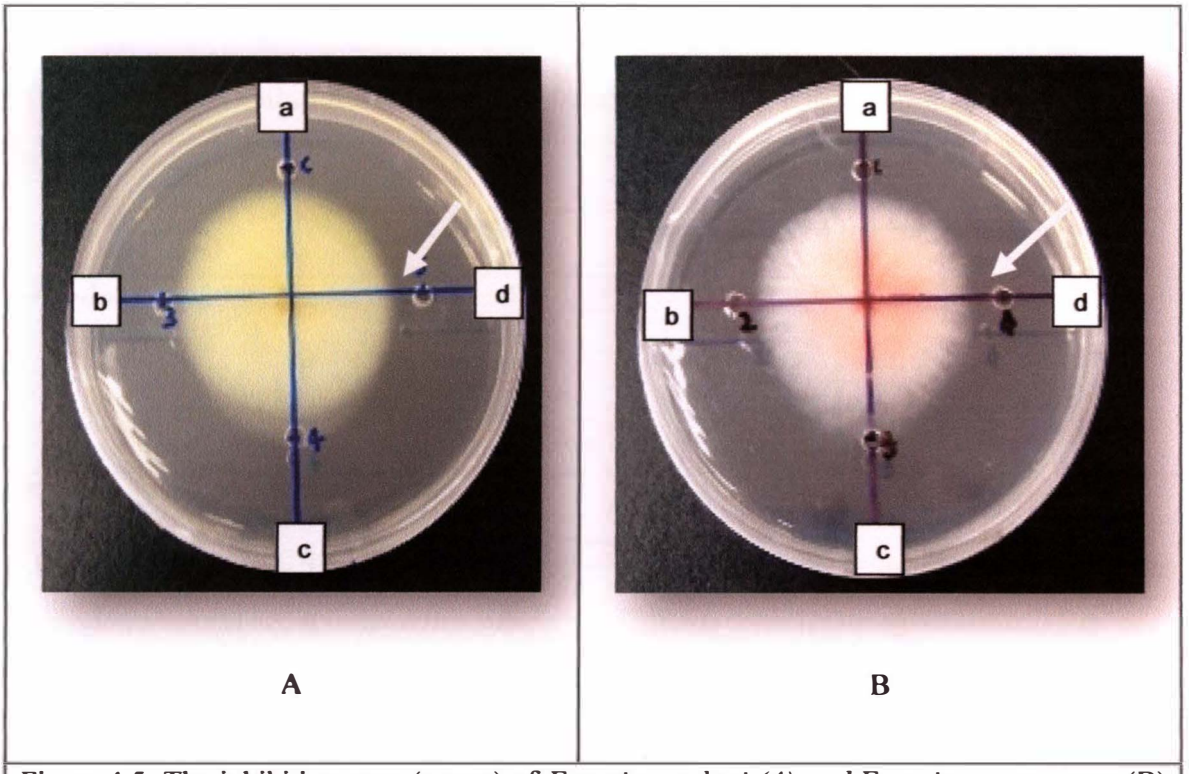


Figure 4.5: The inhibition zone (arrow) of *Fusarium solani (A) and Fusarium oxysporum* (B) on day 6.
*a: control, b: 3 mg/ml, c: 4 mg/ml and d: 5 mg/ml

This technique was performed to overcome the problem of previous method by making a well. So in this technique, cork borer was used to make a well on the agar, extraction concentration had been increased and the fungal colonies had been removed by using a cork borer instead of wire loop. The well can be extent longer retention of extract solution than the paper disc. To obtain a standardized spore, the wire loop was replaced by using cork corer which could give more accurate measurement. Besides, the increasing of extract concentrations can improve the inhibitory results. However, the higher extract volume needed in this method which 100-120 µl compare to only 10-15µl by paper disc. So, this method is not economical to be applied for antifungal studies.

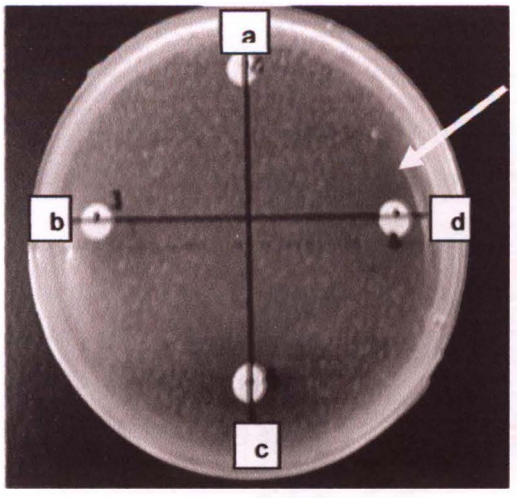
4.4.3 Spore Suspension Technique

Table 4.3: The inhibition zone (mm) of fungi at different garlic extract concentrations

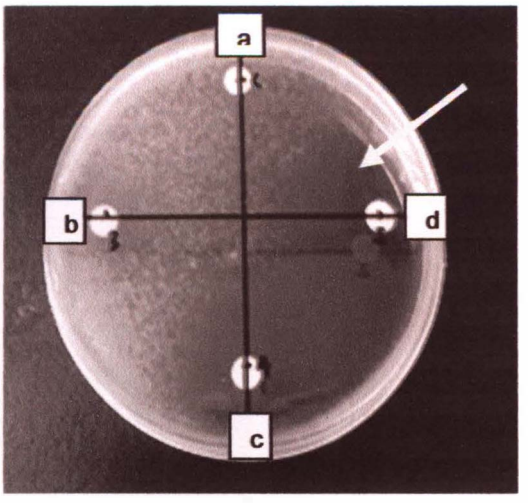
Fungi	Day	Concentrations			
		Control	3 mg/ml	4 mg/ml	5 mg/ml
F. solani	2	0	0	0	2.3
	4	0	0	0	1.3
	6	0	0	0	0
F. oxysporum	2	0	5.7	9.0	14.7
	4	0	1.7	3.7	10.3
	6	0	0	0	0

The spore suspension technique used three concentrations of extract (3, 4 and 5 mg/ml) to inhibit the fungi shown in the (Table 4.3). From the Table 4, only 5mg/ml of garlic extracts had markedly inhibited the development of *F. solani* on day 2 which shown 2.3mm of inhibition zone (Figure 4.6). In contrast, there was no inhibition zone can be observed on negative control (sterilized distilled water), 3 mg/ml and 4 mg/ml.

However, a clear inhibition zone had been obtained in *F. oxysporum* with the 3, 4 and 5 mg/ml of garlic extraction as showed in (Table 4.3 and Figure 4.6). The 5 mg/ml were more effective than other concentrations with largest inhibition were 14.7 mm on day 2 and 10.3 mm on day 4. There was totally no inhibition zone in negative control.



A



B

Figure 4.6: The inhibition zone (arrow) of (A) *Fusarium solani* (5 mg/ml of garlic extraction) and (B) *Fusarium oxysporum* (4 and 5 mg/ml) on day 2.
*a: control, b: 3mg/ml, c: 4mg/ml and d: 5mg/ml

Three methods are been performed to determine the antifungal effect of garlic extraction in different concentration on the *Fusarium solani* and *F. oxysporum*. The method were paper disc diffusion, well disc diffusion and also spore suspension. From the observation throughout this study, the spore suspension technique provided better and clear inhibition zone for both *F. solani* and *F. oxysporum* (Figure 4.6).

For this method same extract concentrations were used by the agar-well diffusion but differed in agar and fungal preparation. Finally, spore suspension method performance showed positive results which overcome the weakness from previous two methods. In this method fungal suspension are prepared by using haemocytometer which allow an accurate determination of volume of fungal spores (Standardized 1 x 10^3 conidial/ml for each replicate). All the spores were distributed

well in the PDA and their growth were more uniform than other technique where the fungi inoculum was placed in the centre of PDA plate (Figure 4.7).

Figure 4.7: The uniform growth of fungal

The paper disc can work effectively in this technique because it had direct contact with the fungal on the agar, and fungal completely retarded when reaction by garlic extract and clear zone had been observed. Improvement of these techniques can overcome the weakness in the first and second methods where fungal need certain time to be in contact with the extract. From this study, the spore suspension technique is the best method to examine the effect of garlic extract on *Fusarium solani* and *Fusarium oxysporum*. Thus, the range between the inhibition zone of garlic extract concentration from this techniques was used as reference to determination of minimal inhibitory concentration (MIC).

4.5 Minimun Inhibition Concentration (MIC)

Table 4.4: Incubation of *Fusarium solani* and *F. oxysporum* with different concentrations ($mgml^{-1}$) for MIC on day 4.

Bijoux bottle	**Concentrations ($mgml^{-1}$)**	***Fusarium solani***			***Fusarium oxysporum***		
		1	2	3	1	2	3
1	10.0	-	-	-	-	-	-
2	5.00	-	-	-	-	-	-
3	4.75	-	-	-	-	-	-
4	4.50	-	+	+	-	-	-
5	4.25	+	+	+	-	+	+
6	4.00	+	+	+	+	+	+
7	Broth	-	-	-	-	-	-
8	Broth Suspension	+	+	+	+	+	+
9	Broth + Extract	-	-	-	-	-	-

*+ = fungal growth; - = no fungal growth

The MIC is defined as the lowest garlic concentration used that completely inhibited visible fungal growth in the broth solutions. Previous study, Fani *et al.*, (2009) mentioned the presence of a white "pellet" and broth colour changing that indicated the susceptibility breakpoint concentration in disk diffusion assay and broth dilution method on garlic extraction. The broth dilution colour changed from white to yellow indicated as the growth of *Fusarium solani* mycelium with conidial in PDB (Figure 4.8A) and the broth dilution colour not showed any changing in 4.50 mg/ml of garlic concentration indicated as no fungal growth (Figure 4.8B). For *Fusarium*

oxysporum, once the bottle was shaked, white "pellet" was observed at the bottom of the bottle showed fungal growth in the broth (Figure 4.9A) and reversely results in the (Figure 4.9B).

Based on the (Table 4.4), the *F. solani* were classified for antifungal susceptibility as follow; susceptible (MIC $\leq$ 4.25mg/ml), intermediate resistant (MIC = 4.5mg/ml) and resistant (MIC $\geq$ 4.75mg/ml). *Fusarium solani* were inhibited with 4.75, 5 and 10mg/ml garlic extraction in PBD where there was no fungal growth. The antifungal susceptibility for *F. oxysporum* as follow; susceptible (MIC $\leq$ 4.00 mg/ml), intermediate resistant (MIC = 4.25 mg/ml) and resistant (MIC $\geq$ 4.50 mg/ml).

A

B

Figure 4.8: The PBD with 1 x 10^3 spore suspension of *Fusarium solani* (A) fungal growth for 4mg/ml (B) no fungal growth for 4.75mg/ml.

A

B

Figure 4.9: The PBD with 1 x 10^3 spore suspension of *Fusarium oxysporum* (A) fungal growth for 4mg/ml (white "pellet" presence at bottom of bottle when shaking) (B) no fungal growth for 4.5mg/ml.

The MIC determination on the effectiveness of the garlic extract was more economical compared to the other in-vitro techniques due to very little amount of sample needed. The MIC can determine the turbidity and presence of white "pellet" in the broth solution for the most minimum concentration of garlic extract.

CHAPTER 5

CONCLUSION

Fungi growth was inhibited by garlic extraction and this can be observed better on the spore suspension technique. The inhibitory action on fungal growth has been attributed to the presence of allicin compound (Muhsin *et al.*, 2000). The allicin as abundant sulfur compound which can present at 10 mg/g in fresh garlic or 30 mg/g in dry weigh (Lawson, 1996). The main thiosulfinate formed upon crushing garlic and it action as fungistatic or fungicidal component that disrupt fungal cell metabolism due to the oxidation of protein (Muhsin *et al.*, 2000). Based on spore suspension technique, *Fusarium solani* was stonger than *F. oxysporum* where the inhibition zones were larger than F. solani on day 2 for 5 mg/ml of garlic extract. The minimal inhibition concentration for *F. solani* was 4.75 mg/ml and *F. oxysporum* was 4.50 mg/ml.

The garlic extract has the potential in the antifungal activities and also inexpensive source of organic compound. Unfortunately, the strong odour caused by the biochemical compounds in the garlic was unfavourable to further utilized as natural food preservatives. The antifungal activities against of garlic extract *Fusarium* species were well observed in in-vitro studies. However, there should be more investigation of the active compounds in the garlic extract and also the antifungal activities towards other common postharvest diseases.

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APPENDIX

Appendix (A)

Potato Dextrose Agar (PDA)

Potato Dextrose Agar powder	39 g
Distilled water	1000 ml

Appendix (B)

Potato Dextrose Broth (PDB)

Potato Dextrose Broth powder	22 g
Distilled water	1000 ml

Appendix (C)

Water Agar (WA)

Agar	20 g
Distilled water	1000 ml

Appendix (D)

Carnation leaf-piece agar (CLA)

Sterile carnation leaves (5-8mm) about 4 pieces were placed in Petri dish consist of 2% of water agar

Appendix (E)

Mean of the length of the inhibition zones affected by the three inhibition test on *Fusarium solani* and *F. oxysporum* on day 4

Technique	Fusarium solani				Fusarium oxysporum			
	Control	1	2	3	Control	1	2	3
Paper disc	17.00± 0.00 ^{ab}	17.33± 0.58 ^{ab}	17.67± 0.58 ^{b}	16.33± 0.77 ^{a}	19.33± 1.16 ^{a}	20.00± 0.09 ^{a}	19.33± 0.59 ^{a}	18.67± 0.58 ^{a}
Agar-well	11.33± 0.58 ^{b}	11.00± 0.00 ^{b}	10.67± 0.58 ^{ab}	9.70± 0.58 ^{a}	14.33± 0.58 ^{a}	14.00± 1.00 ^{a}	13.00± 0.09 ^{a}	12.67± 0.58 ^{a}
Spore suspension	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	0.00± 0.00 ^{a}	1.33± 0.58 ^{b}	0.00± 0.00 ^{a}	1.67± 0.58 ^{ab}	3.67± 0.58 ^{b}	12.67± 2.52 ^{c}

Appendix (F)

t-Test for Minimum Inhibition Concentration on day 4

Group Statistics

	N	Mean	Std. Deviation	Std. Error Mean
Fusarium solani	2	4.3750	0.17678	0.12500
Fusarium oxysporum	2	4.6000	0.14142	0.10000

	Levene's Test for Equality of Variance		t-test for Equality of Means						
	F	Sig.	t	df	Sig. (2-tailed)	Mean Differance	Std Error Difference	95% Confidence Interval of the Difference	
								Lower	Upper
Equal variances assumed			-1.406	2	0.295	-0.22500	0.16008	-0.91376	0.46376
Equal variance not assumed			-1.406	1.908	0.301	-0.22500	0.16008	-0.91376	0.49656

CURRICULUM VITAE

Name	: Gan Chie Giap
Permanent Address	: PRC 30, Kampung China, 18500 Machang, Kelantan.
Telephone Number	: 012-9522612/019-9960945
E-mail	: cg08_seagan@live.com
Date of Birth	: 14 June 1984
Place of Birth	: Machang
Nationality	: Malaysian
Race	: Chinese
Gender	: Male
Religion	: Buddha
Education	: (2004-2007) University Malaysia Terengganu (UMT) (2002-2003) Sek. Men. Keb. Tanah Merah (1) (1997-2001) Sekolah Menengah Kebangsaan Hamzah Machang (1991-1996) Sekolah Rendah Jenis Kebangsaan (C) Pei Hwa

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