THESIS

BIOLOGICAL CONTROL OF RHIZOME ROT OF GINGER DURING STORAGE BY ANTAGONISTIC MICROORGANISMS AND MEDICINAL PLANT EXTRACTS

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THESIS

BIOLOGICAL CONTROL OF RHIZOME ROT OF GINGER DURING STORAGE BY ANTAGONISTIC MICROORGANISMS AND MEDICINAL PLANT EXTRACTS

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A Thesis Submitted in Partial Fulfillment of
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Chawalert Trikarunasawat 2008: Biological Control of Rhizome Rot of Ginger During Storage by Antagonistic Microorganisms and Medicinal Plant Extracts. Doctor of Philosophy (Plant Pathology), Major Field: Plant Pathology, Department of Plant Pathology. Thesis Advisor: Associate Professor Vichai Korpraditskul, Dr.sc.agr. 96 pages.

Ginger rhizome rot during storage is a major problem of ginger exportation. The controlling of ginger rhizome rot was conducted in *in vitro* and semi-commercial levels via biological control treatments. The pathogen of ginger rhizomes rot during storage was identified as *Fusarium oxysporum*. Forty-seven isolates of fungi and 17 isolates of bacteria from 321 isolates of microorganism showed antagonistic ability on controlling of the pathogenic fungi.

Thirty-one medicinal plants crude extract were introduced to evaluate their efficacy to control *F. oxysporum* only two of them, betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry). Crude extracts at 100,000 ppm gave high percetage of growth inhibition at 78.27 and 65.49 %, compared to 1,000 ppm of Imazalil, respectively. The efficacy of partially purified plant extracts was conducted. The solvent partition with petroleum ether and ethylacetate revealed that active compound in clove had low polarity character compared to betal vine leave, which showed moderate polarity compound.

To enhance the efficacy of antagonistic microorganisms, three compounds, namely D-fructose, $CaCl_2$ and Chitosan, were tested by poisoned food technique on vegetative growth of *F. oxysporum.* Chitosan 0.6 % and $CaCl_2$ 4 % provided the highest percentage of growth inhibition at 75.33 %, and 66.67 %, respectively. Whereas, $CaCl_2$ concentrations of lower than 1 % and all concentrations of D-fructose (0.5 to 4 %) could not inhibit vegetative growth of the pathogen.

Antagonistic *Trichoderma* 'KM20' and 'PD5' and antagonistic bacteria 'BPL1' were tolerated to betal vine leave and clove extracts and responsible to $CaCl_2$, in the studies on controlling of ginger rhizome during storage. The semi-commercial experiment design suggested that antagonistic bacteria 'BPL1' gave the lowest disease index except fungicide Imazalil which was infected 8.4 % of rhizome rot.

Wound healing of ginger rhizome incubated for 48 hrs. under high relative humidity condition was tolerated to *F. oxysporum* infection, whereas the infected rhizome under dry condition was susceptible. The study of different healing and storage conditions revealed that the treatment of healing before wrapping by plastic bag without holes provided the lowest disease index (2.25) compared to the other conditions except fungicide Imazalil (1.25) and provided the lowest percentage of weight loss (2.8 %).

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Student's Signature Thesis Advisor's Signature

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BIOLOGICAL CONTROL OF RHIZOME ROT OF GINGER DURING STORAGE BY ANTAGONISTIC MICROORGANISMS AND MEDICINAL PLANT EXTRACTS

INTRODUCTION

Ginger rhizomes (*Zingiber officiginal* Roscae.) are commonly used as vegetables or spices depending on the age of rhizomes. The young rhizomes are usually used as vegetables, while the old or dry rhizomes use as spices or medicinal plants. Nowadays, ginger rhizomes are used in various kinds of industry such as medicines, ready-made foods and cosmetics, therefore the requirements of ginger rhizomes in world markets are increasing year by year.

Ginger rhizomes widely produce in Asia and few countries in Africa. In Thailand, the productive areas are mainly in the northern part, and there are small productive areas disperses in southern, northeastern and western parts. The major products are exported to several countries, such as United Arab Emirates, Albania, Germany, Denmark, France, United Kingdom, Hong Kong, Japan, Kuwait, Malaysia, Netherlands, New Zealand, Pakistan, Saudi Arabia and USA.. The export value of during 1999-2003 A.D. 702 Millions baths (28,000 Tons), only small amount of products are domestically used (Luangarunlers, 2004).

Rhizome rot during storage or transportation are the important problem on fresh ginger export. During storage period, the rhizomes might be rotten more than 50 % by fungal infection within 3 to 4 weeks, although the storage condition was setting up to the optimum state. The fungi that isolated from infectious rhizomes were reported in different countries by different species such as *Fusarium roseum, Macrophomina phaseolina, Nectria inventa, Trichorus spiralis, Memnoniella echinata, Gleocladium candidum, Fusarium oxysporum, F. equiseti, Netria inventa, Cylindrocladium scoparium, Cylidrocarpon* sp., *F. solania and Pythium ultimum*. (Mehrotra, 1952; Sarma and Nambiar, 1974; Sharma and Joshi, 1976; Mishar and Rath, 1988; Rath and Mishar, 1993; Kim *et al.,* 1998). In Thailand, Tanboon-Ek *et al.*

(1978) found that *F. oxysporum* are responsible for the ginger rhizomes rot during storage.

Generally, the rhizomes, during post-harvest handling processes, were waxed by dipping into a mixture containing fungicide to control ginger rhizome rot before storage. Nowadays, the pesticide residues in the agricultural commodities are issue in food safety aspects by several imported countries. Therefore, the others controlling methods to avoid rhizome rot except fungicides should be considered.

Biological control is an alternative method that encompassed the use of antagonistic microorganism, plant extracts or induced defense mechanism of plant those acts as competitive, antibiosis or disease resistance of plant to the causal pathogens. However, the appropriate antagonistic microorganisms, plant extracts and other inducing methods should be investigated for the proper used methods.

OBJECTIVES

1) To investigate the effective antagonistic microorganism and medicinal plant extracts to control ginger rhizomes rot during storage.

2) To study antagonistic potential of microorganism and antifungal property of plants extracts in inhibitory effects on causal fungi of ginger rhizomes storage rot in both laboratory and storage conditions.

3) To investigate the proper methods of storage conditions to control ginger rhizomes rot during storage.

4) To study the effect of wound healing for controlling ginger rhizome rot during storage.

LITERATURE REVIEW

Ginger rhizomes rot during storage was first reported in India, 1952. Under storage condition, the white mycelium of fungi grew and covered ginger rhizomes. The causal fungus was identified as *Fusarium roseum* and able to infect through the wounds but not in healthy rhizomes. Therefore, the causal fungi that concluded are secondary invader or would invader (Merhotra, 1952).

There were several reports of fungal diseases those infected ginger rhizomes during storage. Sarma and Nambiar (1974) reported that *Macrophomina phaseolona* was causal fungi of ginger rhizome dry rot, which infected both in the fields and storage conditions. Sharma and Joshi (1976) reported that post-harvest diseases and pathogens of ginger rhizomes were occurred in difference kinds of symptoms such as, red rot (*Nectria inventa*), gray rot (*Trichorus spiralis*) and black rot (*Memnoniella echinata*). Mishra and Rath (1988) found that the ginger rhizomes samples collected from local markets in Olissa, India was infected by *Gleocladium candidum*, the pathogens were infected and caused ginger rhizomes rot 15 days after inoculation under inoculation conditions, 25 °C, 100 % RH. Rath and Mishra (1993) found that ginger rhizome samples collected from local markets and ginger fields were infected by *F. oxysporum*, *F. equiseti*, *N. inventa*, *Cylindrocladium scoparium* and *Cylindrocarpon* sp.. In Korea, post-harvest diseases of ginger rhizomes were found in variable symptoms and pathogens, yellow soft rot (*Erwinia carotovora* and *Pseudomonas aeraginosa*), brown rot (*F. solani* and *P. aeraginosa*), localized ring rot (*F. solani*) and water soaked rot (*Pythium ultimum*) (Kim *et al*., 1998).

In Thailand, Tanboon-Ek *et al*. (1978) reported that ginger rhizomes those stored under the experimental storage conditions (13 °C, 80 % RH) for exportation were infected by the pathogen that identified as *F. oxysporum*. In addition, Chantaraotan *et al*. (1986) found that pathogenic fungi of ginger, such as *Pythium* sp., *Sclerotium* sp. and *Fusarium* sp., were isolated from ginger fields, but they are not caused the major losses in ginger cultivation as bacterial wilt of ginger (*P. solanacearum*).

Dohroo (1989) found that infected of ginger rhizome rot, caused by *F. oxysporum* f.sp. *zingiberi*, in the fields was correlated to the infection of ginger rhizomes rot that occurred during storage. In addition, 87 % of infected rhizomes those grown in the fields were rotten before emergence. Rana (1997) reported the consistent results that the development of rotting during storage caused by *F. oxysporum* f.sp. *zingiberi* are slower in rhizomes that infected lately, however, the causal fungi can also be detected on non-infected rhizomes. Kim *et al*. (1998) found that *Pythium myriotylum* could be caused rhizomes rot disease in the ginger fields but small amount of population was detected in storage rhizomes, revealed that *P. myriotylum* were not the virulence causal fungi of ginger rhizomes storage rot.

In addition, increasing of temperature during incubation (15-30 °C) increased a violent infection of others isolates of *Pythium* sp. isolated from infected storage rhizomes. On the others hand, Lana *et al.* (1993) found that rhizomes stored at room temperature (17-25 °C), 40-80 % RH. was infected lesser than the rhizomes stored at lower temperature (13 ±1 °C), 80 % RH. but the water loss was higher in the rhizomes stored at room temperature. Besides, waxing does not utilize to the storage while the rhizomes coated with PVC (polyvinyl chloride) films reduces water loss but it was induced rhizomes rot. Swart and Bzvidenhout (1992) were consistent reported that quality of ginger rhizomes stored at 10 °C was better than rhizomes stored at higher temperature (13-21 °C) but rotten rhizomes was higher by numbering.

The control measures of ginger rhizome rot during storage had been used fungicides, imazalil or prochloraz, effectively control rhizomes rot caused by *Botryodiplodia* sp. *Aspergillus* sp. *Diplodia* sp. *Fusarium* sp. *Rhizoctonia* sp. and *Pythium* sp. at concentration 0.8 g.ai/l incubation temperature 10 °C. (Grech and Swart, 1990). Sharma *et al.* (1992) found that Mancozeb persisted longer than carbendazim in rhizomes steeped in solutions of the fungicides for 60 minutes to prevent storage rots, caused by various fungi. Mancozeb residue was present even after 120 days of storage but that the carbendazim residue was not detected at the same period. The result revealed those Carbendazim-treated rhizomes, compared to mancozeb, has lower health risk when the consumer was peeled out the skin before

consuming. Haware *et al.* (1973) reported aureofungin at 0.02 % and benlate at 0.2 % provided the best control on *F. oxysporum* f.sp. *zingiberi* and did not affect viability of the rhizomes. Mancozeb carbendazim benomyl and IBP showed inhibition potential to the fungi caused ginger rhizomes rot in laboratory but threr was less effect in storage condition when ginger rhizomes was treated IBP aureofungin carbendazim mancozeb and benomyl, respectively. (Sharma and Joshi, 1979) Sharma and Dohroo (1982) reported that soaked ginger rhizomes with 0.1 % of mercuric chloride, 0.2 % of mancozeb or 0.2 % of chlorothalonil before storing could be control the storage rot caused by *Pythium pleroticum* and *F. equiseti*.

Barkai-Golan (2001) mentioned that increased official and public concern about the presence of fungicide residues in foods and the development by pathogens of resistance to major fungicides, are two important reasons for the enhanced interest in the possibility of using biological control as an alternative, non-chemical means of decay suppression. There are many fungicides had banned in recently decade by several countries, FDA of USA banned benomyl in post-harvest commodities and countries in Europe may decides by the same direction eventually. Therefore, the control measures that avoided using fungicides ought to developed, considering to the environments and human health. Biological control may be a control measures that useful to control post-harvest diseases (Chalutz and Droby, 1998). There are many reports on antagonistic microbes using for post-harvest diseases, Mukherjee and Raghu (1997a) found that *Trichoderma* sp. prevented rotting of beat and carrot caused by *Sclerotium rolfsii* during storage and transportation. Dohroo and Sharma (1984) reported that ginger rhizomes rot during storage caursed by *Pythium pleroticum* (wet rot) was reduced 80 % of control by using antagonistic *T. viride*. But Mukherjee Raghu (1997b) found that storing temperature lower than 35 °C *Trichoderma* sp. could not control *Sclerotium rolfsii* in ginger rhizomes. Phillip *et al.* (1999) reported those isolates of antagonistic fungi from healthy tomatoes and cucumbers, which collected from the sources of vegetables that infected by *F. solani Rhizoctonia solani Mucor* spp. *Colletotrichum gloeosporioides Alternaria solani Phytophthora* sp. and *Pythium aphanidermatum*.

Natural antagonistic activities of yeast may disclosures by the multiplication of non-target fungi of benomyl such as *Coahiobolus* spp. *Alternaria* spp. after using of it. On rye leaves, after spraying of benomyl, populations of yeast *Sporobolomyces* spp. was 10 times decreased by control treatment comparison. After that, the infection of *Cochiobolus sativas* was 3 times increased after inoculation compared to control treatments (Jurken and Nyckle, 1997). Schisler *et al.*, (1995) reported that dry rot disease of potatoes during storage caused by *F. sambucinum* and *F. solani* var. *coeruleum* was significantly control by yeast *Cryptococcus laurentii*. Mehrotra *et al.* (1996) found that *Penicillium digitatum* and *P. italicum* on oranges fruits during storage were controlled by dipping of oranges fruits into cells suspension of yeast *Debaryomyces hansenii* before inoculation. However, the sterilized culture filtrate of yeast could not control those pathogens revealed that yeast did not produced antibiotics. Cheah *et al*. (1995) selected the antagonistic yeast from industrial yeast for controlling post-harvest disease of lemon caused by *P. digitatum*, and they found that one isolate of *Sacharomyces cerevisiae* and three isolates of *Kluyveromyces* spp. had potential to reduce the disease.

Costa *et al.* (1997) found that three isolates of non-fluorescence *Pseudomonas* spp., BS1-3 BS1-6 and BS1-12 were potential antagonists against *Collectotrichum musae*, causal fungi of antracnose disease of bananas by dipping of cells suspension of each bacterial isolate.

To enhance the disease control efficiency of antagonists, cells suspension of yeast *Pichia guilliermondii* strain US-7 added 136 mM of $CaCl_2$ was significantly increased the potential to control gray mold (*Penicillium digitatum*) in grapefruit during storage (Droby *et al.,* 1997). While merely using of yeast *Candida sake* strain 13L to control *P. digitatum* was better than adding 2, 4 and 6 % of $CaCl_2$, more over, it was toxic to yeast (Arras *et al.,* 1997). Piano *et al*. (1997) found that nutrients are main factors of the potential of yeast *Metschnikowia pulcherrima* to inhibit fruit rot of apples caused by *Botrytis cinnerea*, the experiments shown that treatment added 100 g/l of fructose were increased control efficiency to the pathogen. Ghout *et al*. (2000a) reported that combined using of *Candida saitoana* and 0.2 % of glycochitosan gave higher efficiency to control decay during storage of apple fruits than merely used.

Ghout *et al.* (2000b) found that combined using of *C. saitoana* and 0.2 % of 2-Deoxy-D-Glucose before inoculation gave the best efficiency to control decaying of apples lemons and oranges during storage compared to merely used. However, using of the combination after inoculation 24 hr gave inhibited on blue mold of apples, green mold of oranges and lemons equal to using of imazalil. 2-Deoxy-D-Glucose that added into the culture of *C. saitoana* had affected on growth rate of the yeast but the wound experiment showed that either of presents or absences of 2-Deoxy-D-Glucose dose not affects to growth rate of *C. saitoana*.

McGuire and Bolwin (1994) found that cellulose base coating agents affords growth of yeast such as *C. albidus Rhodotola mucilaginosa* and *Cryptococus albidus* on grapefruit skins. Pogewijd *et al.* (1995) reported consistence results on combining use of methyl cellulose base coating agent and cells suspension of *C. guilimondii* strain US7 were significantly decreased natural decay of pineapples and Valencia oranges at storing periods 2-4 weeks, temperature 10 °C, 90 % RH. The results shown that efficiency of the mixtures were closed to the commercial coating agent at concentration 2000 mg/l, which added reducing decay chemicals.

Herbs or spices also reported that inhibited growth of fungi. Soytong (1985) found that twenty from twenty-one isolates of fungi was 100 % inhibited in the experiment that conducted by poisoned food techniques with the extracts of Star anise (*Illicium verum* Hook.f. *ExT.*) And To-anun (1985) reported that inhibitory effect of ground dry leaves of *Piper betal* added into the medium of twelve species of *Aspergillus* spp. Sinchaisri *et al.* (1992) reported that 0.5 % of Prayong (*Ageratina odorata* Loua.) and Hanuman prasan kai (*Schefflera ieucantha* R. Vig. S.) inhibited five pathogenic fungi, namely *Alternaria* sp., *Macrophomina* sp., *Fusarium* sp., *Phomopsis* sp., *Phytophthora* sp., *Colletotrichum* sp. and *Sclerotium* sp.. Ejechi *et al.* (1997) found that growing medium added of 5 extracts of Nigeria's spices, ginger garlic *Aframomum nuleguta, Xylopia aethiopica* and *Monodora myristica* inhibited growth of *Botryodiplodia threobromae, Aspergillus niger, A. flavus, Mucor* sp., *Rhizopus stolonifer, Penicillium* sp. those isolated from rotten okra pods. Only ginger and *Monodora myristica* extracts were able to decreased rotting of okra pods by

combined using with low heat treatment, after 3 months of storing period revealed that only 0.2 % of carbohydrate in okra pods were lossed.

MATERIALS AND METHODS

Materials

1. Medicinal Plant Materials

Table 1 Medicinal Plant Materials

No.	Thai names	Common names	Scientific names
1	Boraphet	-	*Tinospora crispa* Miers
2	Bua bok	Asiatic Pennywort	*Centella asiatica* (L.) Urban.
3	Chumhet thet	Candelabra Bush	*Cassia alata* Linn.
4	Fa thalaai	-	*Andrographis paniculata* (Burm.) Wall. Ex. Nees.
5	Hanumaan prasaan khai	-	*Scheffera* sp.
6	Kaan phluu	Clove	*Eugenia aromaticum* (Linn.) Merr & Perry.
7	Kaeo	China Box Tree	*Murraya paniculata* (linn.) Jack.
8	Kek-huai	Asiatic Pennywort	*Chrysanthemum indicum* Linn.
9	Kham foi	Safflower	*Carthamus tinctorius* Linn.
10	Khamin chan	Turmeric	*Curcuma domastica* Val.
11	Kheelek	Thai Copper Pod	*Cassia siamea* Lamk
12	Khing	Ginger	*Zingiber officinale* Roscoe
13	Khluu	Indian Marsh Fleabane	*Pluchea indica* Less.
14	Krachiap daeng	Roselle	*Hibiscus sabdariffa* Linn.
15	Krathiam	Garlic	*Allium sativum* Linn.
16	Luuk tai bai	-	*Phyllanthus virgatus* Forst.
17	Mara khee nok	Bitter Cucumber	*Momordica charantia* Linn.
18	Matuum	Bael Fruit Tree	*Aegle marmelos* (L.) Corr.

Table 1 (Continued)

No.	Thai names	Common names	Scientific names
19	Mon	Mulberry tree	*Morus alba* Linn.
20	Op choei	Cinnamon tree	*Cinnamomum zeylanicum* Linn.
21	Phet sangkhaat	-	*Cissus quadrangularis* Linn.
22	Phluu	Betal	*Piper betal* L.
23	Pooi kak	-	*Illicium verum* Hook.
24	Prayong	-	*Aglaia odorata* Lour.
25	Raang chute	-	*Thunbergia laurifolia* Linn.
26	Som khaek	-	*Garcenia atroviridis* Griff.
27	Ta khrai	Lemon Grass	*Cymbopogon citratus* Stapf
28	Yaa dok khaao	-	*Vernonia cinerea* Less.
29	Yaa haeo moo	Nut Grass	*Cyperus rotundus* Linn.
30	Yaa khaa	-	*Imperata cylindrica* Beauv.
31	Yaa nuat maeo	-	*Orthosiphon grandiflorus* Bolding.

2. Cold Room

Cold room (12.5 °C)

3. Fungicides and chemical materials

3.1 Imazalil (Imazalil®)

3.2 Chitosan (Seafresh Chitosan (Lab) CO., LTD.; 84 % DAC

3.3 $CaCl_2$

3.4 D-fructose

5. Fungi

Fusarium oxysporum

6. Equipment

6.1 Rotary evaporator
6.2 Shaker
6.3 Universal Hardnessmeter (KIYA® 1 kgUB)
6.4 Micropipette
6.5 Homogenizer

7. Laboratory Materials

7.1 Petri dish
7.2 Flask
7.3 Beaker
7.4 Separate funnel
7.5 Buckner funnel
7.6 Alcohol
7.7 Loop
7.8 Needle
7.9 Sodium hypochlorite (Clorox®)
7.10 Tube
7.11 Mixer
7.12 Cork borer (0.5 cm diameter)
7.13 Pipette
7.14 Autoclave
7.15 Hot air-oven
7.16 Heamacytometer
7.17 Filter paper
7.18 Vacuum desiccator
7.19 Vacuum pump
7.20 Moist chamber (95-100 % RH)

8. Microscopes

Stereo Compound Microscope

9. Antibiotics

Streptomycin

10. Organic Solvents

10.1 Ethanol
10.2 Petroleum ether
10.3 Ethyl acetate

Methods

1. The isolation of pathogen and antagonistic microorganism and the Preparation of Plant extracts.

1.1 Isolation of Pathogen.

Infected ginger rhizomes during storage were used for isolation of causal organism by using tissue-transplanting technique (Dake and Edison, 1989). Ginger tissues at the boundaries of lesion and healthy tissue were collected. The ginger tissue surrounding the infected area was cut for small pieces, 0.5 x 0.5 mm, and soaked with 1 % sodium hypochloride for 5 minutes, then rinsed 3 times with steriled water and placed on the surface of PDA (Potato Dextrose Agar) plates. Seven days after incubation period, the fungal mycelia that germinated from ginger tissues were reisolated and sub-cultured on PDA slants. The fungus that caused ginger rhizome rot during storage was identified as *Fusarium oxysporum* (Nelson *et al*., 1983) (Figure 1).

1.2 Pathogen inoculum preparation.

The spore suspension of the pathogen was prepared by harvesting conidia of *Fusarium oxysporum* from 10-day-old PDA culture and washed with 10 ml of steriled water. The concentration of spore suspension was adjusted to 10^4 cfu by spore counting under hemacytometer.

The agar plugs of 0.5 cm diameters were prepared by spreading 0.1 ml spore suspension of pathogens (*Fusarium oxysporum*) onto PDA Petri dishs (9 cm diameter) incubated for 3 days at room temperature (28-30 ºC). An agar plug was cut by 0.5 cm diameter cork borer thereafter they were transferred on to the PDA plates.

1.3 Isolation of antagonistic microorganism and screening for antagonists.

Ginger rhizomes, root and soil samples were collected from 12 ginger planting-area in 3 different parts of Thailand; there were 9, 1 and 2 fields from Northern, Northeastern and Southern, respectively (Table 2).

Table 2 Ginger rhizome plantations those are rhizomes, root and soil samples were collected.

Regions		Plantations
Northern	1	Baan Khokmon, Amphur Nhamnao, Phetchaboon
	2	Baan Huay Sai Nua, Tumbol Huay Sai Amphur Nakhonthai, Phitsanulok
	3	Baan Huay Sai Tai, Tumbol Huay Sai, Amphur Nakhonthai, Phitsanulok
	4	Baan Huay Makang, Tumbol Pha Dad, Amphur Saruay, Chiang Rai
	5	Tumbol Mae Phlu Luang, Amphur Wiang Pha Pao, Chiang Rai
	6	Location between Amphur Wiang Pha Pao to Amphur Prao, Chiang Mai
	7	Baan Pha Bong Ngam, Tumbol Muang Na, Amphur Chiang Dao, Chiang Mai
	8	Tumbol Sob Pong, Amphur Pang Ma Pha, Mae Hong Son
	9	Amphur Ngaw, Lampang
Northeastern	10	Baan Keang, Tumbol Rong Jik, Amphur Phu Ruoe, Loei
Southern	11	Baan Huay Num Kheuw, Tumbol Ron Thong, Amphur Bang Saphan, Prachuap Khiri Khan
	12	Tumbol Thummarat, Amphur Bang Saphan, Prachuap Khiri Khan

1.3.1 Microbial isolation from soil samples.

Soil samples were collected from ginger fields at 10 cm depth from soil surface surrounding root zone, and microbials were isolated by using dilution plate technique (Dhingra and James, 1995) at dilution 10^{-3} – 10^{-4}, 0.1 ml. of each dilution was dropped on the surface of semi liquid medium in a Petri dish and spread with grass rod. PDA medium was added with 100 µg/l of streptomycin and 50 µg/l of rose-bengal as selective media for fungal isolation and Nutrient Agar (NA) plate was used for bacterial isolation. The Petri dish were incubated at room temperature (28-30 ºC) for 3-7 days. Each single colony of fungi and bacteria were collected and reinoculated on PDA or NA slants.

1.3.2 Microbial isolation from ginger rhizomes.

The healthy ginger rhizomes were shake with steriled water for 30 minutes, and then the rhizomes skin were peeled off. The washing water portions were isolated by using dilution plate technique. The skins of ginger rhizomes were cut for small pieces (approximately 5 x 5 mm), 5 pieces were placed on the surface of PDA plates added with 100 µg/l of streptomycin and 50 µg/l of rose-bengal as selective media for fungi isolation and NA plates for bacterial isolation.

1.3.3 Microbial isolation from water of ginger rhizomes rinsed.

Rinsed water of ginger rhizomes from 1.3.2 were isolated the microorganism by dilution plate technique and thereafter they were incubated and reisolated for pure culture in 1.3.1.

Three hundreds and twenty-one isolates of microorganism were isolated, 193 isolates were fungi and 128 isolates were bacteria. All microbes were delivered to test for their antagonistic ability to *Fusarium oxysporum*, a ginger rhizome rot pathogen.

1.4 Preparation of plant extracts and partially purified fractions.

1.4.1 Preparation of plant extracts (Wangkiat, 1995).

Thirty-one species of herbs and spices were selected to test for vegetative growth inhibition ability to the pathogen. The plant parts were chopped and air-dried for 3 days before ground. Ten gm of each sample introduced to 500 ml of flask, and then 95 % ethanol were added into the flask until the level of ethanol over the surface of samples, incubated at room temperature (28-30 °C) under dark condition for 3 days. The filtrates were filtered by the Whatman No1 filter paper, the residues of samples were re-extracted 2nd and 3rd extraction with the same method. All filtrates were combined and then removed ethanol by using vacuum rotary evaporator at 45 °C and dried out in vacuum desiccators. The dry crude extracts weight was calculated for making dilutions. (Table 3)

Table 3 Thai medicinal plants.

No.	Thai names	Common names	Scientific names	Plant Part
1	Boraphet	-	*Tinospora crispa* Miers	Leave and stem
2	Bua bok	Asiatic Pennywort	*Centella asiatica* (L.) Urban.	Leave
3	Chumhet thet	Candelabra Bush	*Cassia alata* Linn.	Leave
4	Fa thalaai	-	*Andrographis paniculata* (Burm.) Wall. Ex. Nees.	Leave and stem
5	Hanumaan prasaan khai	-	*Scheffera* sp.	Leave
6	Kaan phluu	Clove	*Eugenia aromaticum* (Linn.) Merr & Perry.	Flower
7	Kaeo	China Box Tree	*Murraya paniculata* (linn.) Jack.	Leave
8	Kek-huai	Asiatic Pennywort	*Chrysanthemum indicum* Linn.	Flower
9	Kham foi	Safflower	*Carthamus tinctorius* Linn.	Flower
10	Khamin chan	Turmeric	*Curcuma domastica* Val.	Rhizome
11	Kheelek	Thai Copper Pod	*Cassia siamea* Lamk	Leave
12	Khing	Ginger	*Zingiber officinale* Roscoe	Rhizome
13	Khluu	Indian Marsh Fleabane	*Pluchea indica* Less.	Leave
14	Krachiap daeng	Roselle	*Hibiscus sabdariffa* Linn.	Flower
15	Krathiam	Garlic	*Allium sativum* Linn.	Bulb
16	Luuk tai bai	-	*Phyllanthus virgatus* Forst.	Leave and stem
17	Mara khee nok	Bitter Cucumber	*Momordica charantia* Linn.	Fruit
18	Matuum	Bael Fruit Tree	*Aegle marmelos* (L.) Corr.	Fruit

Table 3 (Continued)

No.	Thai names	Common names	Scientific names	Plant Part
19	Mon	Mulberry tree	*Morus alba* Linn.	Leave
20	Op choei	Cinnamon tree	*Cinnamomum zeylanicum* Linn.	Bark
21	Phet sangkhaat	-	*Cissus quadrangularis* Linn.	stem
22	Phluu	Betal	*Piper betal* L.	Leave
23	Pooi kak	-	*Illicium verum* Hook.	Pod
24	Prayong	-	*Aglaia odorata* Lour.	Leave
25	Raang chute	-	*Thunbergia laurifolia* Linn.	Leave
26	Som khaek	-	*Garcenia atroviridis* Griff.	Fruit
27	Ta khrai	Lemon Grass	*Cymbopogon citratus* Stapf	Leave
28	Yaa dok khaao	-	*Vernonia cinerea* Less.	Leave
29	Yaa haeo moo	Nut Grass	*Cyperus rotundus* Linn.	Rhizome
30	Yaa khaa	-	*Imperata cylindrica* Beauv.	Rhizome
31	Yaa nuat maeo	-	*Orthosiphon grandiflorus* Bolding.	Leave

1.4.2 Partially purification compounds (Wangkiat, 1995)

Selected plant extracts which showed inhibitory effects were partially purified by solvent partition with petroleum ether and ethyl acetate in order to separate the polarity of active compounds into low-polarity compounds and the moderate-polarity compounds, respectively.

2. Screening for antagonists and the efficacy test of plant extracts.

2.1 *In vitro* testing for antagonistic ability.

Fungi isolates from soil and ginger rhizome samples were introduced to test for antagonistic ability by spot inoculation test (Inwang and Chamswang, 1986). An agar disc of each isolate of soil microbe and *F. oxysporum* was placed on PDA

plates, incubated for 3-5 days at room temperature (28-30 °C), then agar with actively mycelium was cut by 0.5 cm diameter cork borer and they were carried out for testing. Agar plugs with pathogen, *F. oxysporum*, were prepared by the procedures as mentioned in '1.2 Pathogen inoculums preparation' section 1. An agar plug of tested fungal mycelium was placed 6 cm apart from another agar plug with pathogen mycelium on PDA surface in a petri dish.

Bacterial isolates were tested for antagonistic ability by using filter paper disc method (Dingra and James, 1995). Bacterial cell cultures were prepared by inoculating bacterial isolates into NB (nutrient broth) medium, incubated for 48 hrs. PDA plates were prepared and a pathogen agar plug (*F. oxysporum*) was placed at the center. A sterilized filter paper disc was dipped in bacterial isolate cell cultures, and then placed surrounding the center, with 3 cm spacing from the pathogen agar plugs. A petri dish was tested for the 4 bacterial isolates and incubated at room temperature (28-30 ºC). Inhibition zone (clear zone) surrounded bacterial paper disc was observed and suggested its antagonistic ability.

2.2 Testing for growth of antagonists on ginger rhizomes.

Fungal and bacterial isolates that showed antagonistic interaction to the pathogen (*F. oxysporum*) were introduced to test for growing on ginger rhizomes. Ginger rhizomes were prepared by washing with tap water, and then cut the rhizomes with sterilized knife to make wounds. The rhizomes were soaked with 5 % Clorox for 10 minutes, rinsed 3 times with distilled water and let air-dry at room temperature. Cells or spores suspension of antagonistic fungi and bacteria were prepared from cultures of each isolate of antagonist. Two isolates of fungi namely *Trichoderma* isolate 'KM20' and 'PD5' and two isolates of bacteria namely isolate 'BMJ7' and 'BPL2'were selected for this experiment. Spores suspension of antagonistic fungi were prepared from fungal PDA cultures, 3-7 days old, Ginger rhizomes were dipped into cell or spore suspension and incubated for 16 days at room temperature, 95-100 % RH.

A Population of antagonist was detected on ginger rhizome tissues. Both skin and wound areas of rhizomes were cut by cork borer, three pieces of tissue were ground with homogenizer in 10 ml of steriled water. The tissue suspension was diluted to 10^{-2}-10^{-4} and 0.1 ml of each dilution was pipetted and spread onto agar plates. Selective media were used to specify the antagonists. The colonies of antagonist microorganism were counted and calculated log of cfu/ml at 0, ½ , 1, 2, 4 and 8 DAI (Days After Inoculation). Firmness of tissue of skins and wounds area was measured by Universal Hardnessmeter (KIYA® 1 kgUB) at 0, ½, 1, 2, 4, 8 and 16 DAI.

2.3 Testing for the efficacy of culture filtrate of antagonistic microorganism on vegetative growth of pathogen.

The culture filtrates of antagonistic *Trichoderma* isolate 'KM20' and 'PD5' and antagonistic bacteria isolate 'BMJ7' and 'BPL2' were introduced to test by filter paper disc method on PDA medium. The clear zone of pathogen was measured and recorded.

2.4 Testing for the efficacy of plant extracts on vegetative growth of pathogens.

2.4.1 Testing for inhibitory effect of plant extracts on vegetative growth of the pathogens.

Filter paper disc technique was employed to screen plant extracts that showed inhibitory effect on vegetative growth of the pathogen (*F. oxysporum*). Each plant extract from section 4.1 was carried out to dissolve by 95 % ethanol and made diluted to 100,000, 50,000 and 10,000 ppm by micropipette, 10 µl of each plant extracts dilution was dropped on 0.5 cm diameter sterilized filter paper disc and let air-dry for 3 minutes, dropping of plant extract was done by 3 times on each paper disc. Positive and negative control treatments were dropped by 10 µl of 1,000 ppm imazailil and 95 % ethanol, respectively. Diameter of inhibition zone (clear zone) surrounded paper disc was measured for 5 days, at 24 hr interval.

2.4.2 The efficacy of partially purified plant extracts.

Plant extracts of betal vine leave (*Piper betal* L.) and dry flower of clove (*Syzygium aromaticum* (Linn.) Merr & Perry.), that showed inhibition effect on vegetative growth of pathogens (*F. oxysporum*) were employed partially purification using for solvent partition with petroleum ether and ethyl acetate as the procedures mentioned in section 1.4.2 to indicate polarity of active compound. All fractions of plant extracts were made dilution at 100,000, 50,000 and 10,000 ppm and used for testing growth inhibition of pathogens using filter paper disc technique. The fraction that showed inhibitory effect on growth of pathogens was suggested appearance of active compounds.

2.5 The efficacy of antagonists and plant extracts on controlling ginger rhizome rot (*Fusarium* rot) during storage.

2.5.1 The efficacy of antagonists on controlling of ginger rhizome rot was conducted on ginger rhizomes, the procedures was prepared as mentioned in section 2.2. The rhizomes were soaked with spore suspension of antagonists (prepared by the procedures as mentioned in section 1.2) for 3 minutes. Let the rhizomes air-dry at room temperature, then sprayed with spore suspension of *F. oxysporum*. The ginger rhizomes were incubated for 16 days at room temperature, 95 % RH.

Populations of antagonist and pathogens (*F. oxysporum*) were detected on ginger rhizome tissues. Both skin and wounded areas of rhizomes was punctured by cork borer. Three pieces of tissue were ground with homogenizer in 10 ml of steriled water. The tissue suspensions were made dilution at 10^{-2}-10^{-4} and 0.1 ml of each dilution was pipette and spread onto agar plates. Selective media were used to specify the antagonists and *F. oxysporum* (*Trichoderma* selective medium I and *Fusarium* selective medium V (Nash & Snyder medium)) (Dhingra and James, 1995). The colonies of antagonist and *F. oxysporum* were counted and calculated as log of cfu/ml at 0, ½, 1, 2, 4 and 8 DAI (Days after inoculation). Firmness of tissue of skins

and wounded areas were measured by Universal Hardnessmeter (KIYA® 1 kgUB) at 0, ½, 1, 2, 4, 8 and 16 DAI.

2.5.2 The efficacy of plant extracts on controlling of ginger rhizome rot was conducted on ginger rhizomes and the procedures was prepared as mentioned in section 2.2. The rhizomes were soaked with plant extracts at 10,000 ppm in distilled water, let it air-dry, then sprayed with spore suspension of *F. oxysporum*. The rhizomes were incubated for 16 days at room temperature, 95 % RH..

A population of pathogen (*F. oxysporum*) was detected on ginger rhizome tissues. Both skin and wounded areas of rhizomes were punctured by cork borer, three pieces of tissue were ground with homogenizer in 10 ml of steriled water. The tissue suspensions were made dilutions at 10^{-2}-10^{-4} and 0.1 ml of each dilution was pipetted and spread onto agar plates. Selective media were used to specify *F. oxysporum* (*Fusarium* selective medium V (Nash & Snyder medium)) (Dhingra and James, 1995). The colonies of *F. oxysporum* were counted at 8 DAI (Days after inoculation)and calculated as log of cfu/ml at 0, ½, 1, 2, 4 DAI. Firmness of skins tissue and wounded tissue were measured by Universal Hardnessmeter (KIYA® 1 kgUB) at 0, ½, 1, 2, 4, 8 and 16 DAI.

3. The examination of some compounds mixed up with plant extract and antagonistic microorganism to control ginger rhizome rot.

3.1 *In vitro* screening of some compounds on vegetative growth of the pathogen.

The poisoned food technique was employed to test the inhibition of vegetative growth of pathogen (*F. oxysporum*). The enhancing compounds used in the experiment were D-fructose at 0.5, 1.0, 2.0 and 4 %, CaCl2 at 0.5, 1.0, 2.0 and 4 % and Chitosan (Seafresh Chitosan (Lab) CO., LTD.; 84 % DAC ; in 1 % acetic acid) at 0.1 ,0.2, 0.3, 0.4 and 0.6 %. Each of compounds and concentrations was added into the melting PDA at 60 ºC, mixed and poured into petri dish. The fungicide Imazalil 1000 ppm and distilled water were used as control treatments. An agar plug with 2

days old fungal mycelium was placed at the center in a PDA plate. Diameter of fungal colony was measured and calculated to % growth inhibition of each chemical using the formula as follow:

$$\% \text{ Growth inhibition} = \frac{A - B}{A} \times 100 \quad \text{when}$$

A = Colony diameter of control – 0.6 (cm)
B = Colony diameter of treatment - 0.6 (cm)

3.2 *In vitro* testing of some compounds on plant extracts on growth of antagonistic microorganism.

To evaluate the response of antagonists to some compounds and plants extracts, poisoned food technique and filter paper disc method were employed to test the vegetative growth inhibition and clear zone that produced by antagonistic microorganism.

Testing for the response of antagonistic bacteria and fungi to plant extract, filter paper discs (0.5 cm. diameter) were dipped into crude extract of betal vine leave and clove at 50,000, 100,000 ppm, and let air dry. Then placed on the surface of media, Nutrient Agar or PDA in Petri dish, which spread with cell suspension of antagonistic bacteria or fungi.

Testing for the response of antagonistic bacteria and fungi to some compounds, the PDA plus compounds were 2 and 4 % of $CaCl_2$, 0.3 and 0.6 % of chitosan and 2 and 4 % of D-fructose were prepared in Petri dish. A paper disc (0.5 cm. diameter) dipped antagonistic bacteria cell suspension or an agar disc (0.5 cm. diameter) with active mycelium of antagonistic fungi was placed on the surface of PDA.

3.3 Control of ginger rhizome rot during storage by using antagonistic microorganism with enhancing compounds and plant extracts.

The antagonistic bacteria ‘BPL2’ and antagonistic Trichoderma ‘KM20’ ‘PD5’with enhancing compounds, Chitosan, $CaCl_2$ and clove and betal vine leave extracts were used in combination for controlling of ginger rhizome rot during storage. The ginger rhizome was washed with tap water, expose to the air until dry, and then cut for make wound surfaces. Treatments included 2,000 ppm of betal vine leaves and clove extracts, 2 % of $CaCl_2$, and 0.3 % of Chitosan. After soaking the ginger rhizomes with those treatment solutions, were let air dry under the room temperature (28±2 ºC) for 12 hr. One kilogram of treated ginger rhizome was packed into a card box. Three card boxes were conducted for replication of each treatment. The ginger rhizome were stored in low temperature (13 ºC) for 4 weeks. The ginger rhizome was brought out for visually observed and recorded the Disease Index (DI : 1-10 ; 1= 100 % healthy-10 = 100 % rotten).

The results of first experiment were analyzed and decided to discard treatments those provided the poor results. The rest treatments were 0.3 % of chitosan, 2000 ppm of Clove extract, 1,000 ppm of Imazalil, antagonistic bacteria ‘BPL2’, 0.3 % of chitosan + 1,000 ppm of Imazalil and Control (DW).The experiment was conducted as the same measure.

The experiment has been done by semi-commercial condition using 10 kg. of ginger rhizome in card box. The discarded treatments were 2000 ppm of Clove extract, 1,000 ppm of Imazalil, antagonistic bacteria ‘BPL2’ and Control (DW). The experiment was conducted in 3 replications. Disease index and weight loss were measured and recorded.

4. Influencing factors of inducing wound healing on ginger for controlling of rhizome rot during storage.

4.1 Effect of healing conditions on rhizome rots control.

The ginger rhizomes were cut to small pieces to make the wounds, and kept them under different healing conditions combining between, 3 levels of

temperature, 15 ºC, 28±3 ºC (Room Temp.) and 32 ºC and high/low humidity condition (open air and moist chamber) incubated for 48 hr. Then the ginger rhizomes were inoculated with *Fusarium oxysporum* spore suspension which was prepared as mentioned in 1.2, and then incubated in moist chamber at room temperature for 8 days. The infectious symptoms were recorded.

4.2 Controlling of ginger rhizome rot during storage by using healing conditions.

The ginger rhizome was prepared as mentioned in 4.1 and the treatments were combined between healing (H^+), not healing (H^-) treated and put into plastic bag with/without holes compared to 1,000 ppm imazalil fungicide dipped sample. Disease Index (0-9 ; 0=healthy 9= heavily infection) and weight loss were recorded at 7 days interval for 6 times (42 days). The experiment was conducted under storage condition at 13 °C 65 % RH.

RESULTS AND DISCUSSIONS

Results

1 The isolation of pathogen and antagonistic microorganism and the Preparation of Plant extracts.

1.1 Isolation of Pathogen.

Twenty-six isolates of fungi were isolated from infected storage rhizomes (Figure 1 A and B), but only one isolate could infect the healthy rhizome (Figure 1 C). This isolate was subculture to PDA in Petri dish and made permanent slides. The fungal isolate was characterized and identified as *Fusarium oxysporum*.

1.2 Pathogen inoculum preparation.

Pure culture of *Fusarium oxysporum* was subculture to PDA plates, seven-day-old culture was used for preparing pathogen spore suspension. Agar block culture, 0.5 mm diameter was prepared by cutting 7 days-old culture on PDA with sterilized cork-borer.

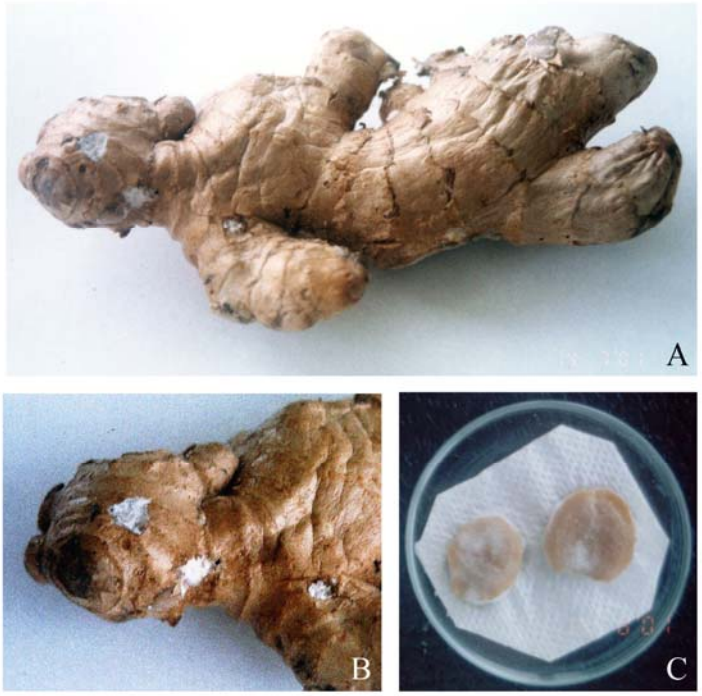

Figure 1 Ginger rhizomes rot symptoms on fresh ginger rhizomes during storage (A and B) and the isolated pathogenic fungus, *Fusarium oxysporum*, could be grown on ginger rhizomes (C).

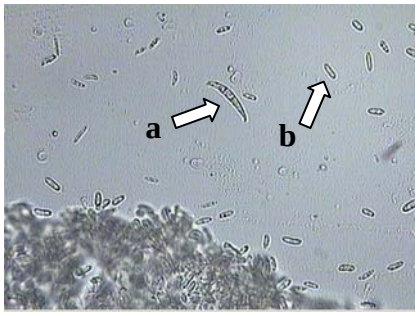

Figure 2 The macroconidium (a) and microconidium (b) of *Fusarium oxysporum* under light microscope (x200)

1.3 Isolation of antagonistic microorganism and screening for antagonists.

Soil samples around the root zone (rhizospere) and rhizome of ginger were collected from different twelve planting areas in three parts of Thailand. Nine planting areas from the northern part, one planting area from northeastern part and two planting areas from southern part (Table 4).

Soil microorganism were isolated by soil dilution plate method, while the organism on the surface of ginger rhizome was isolated from washed water. All samples were made dilution series and mixed with warm steriled PDA. Three days after incubation, single colony of fungal and bacteria were reisolated on PDA plate and slant PDA, respectively. One hundred ninety-three isolates of bacteria and 128 isolates of fungi were obtained, and thereafter they were evaluated their antagonistic properties against *F. oxysporum* under labolratory and practical used, respectively.

Table 4 Ginger-planting areas those are rhizomes, root and soil samples were collected from 3 different parts of Thailand and the numbers of microorganism isolates obtained in each location.

Plantations	No. of isolates	
	Fungi	Bacteria
Northern part		
1. Baan Khokmon, Amphur Nhamnao, Phetchaboon	40	14
2. Baan Huay Sai Nua, Tumbol Huay Sai Amphur Nakhonthai, Phitsanulok	22	14
3. Baan Huay Sai Tai, Tumbol Huay Sai, Amphur Nakhonthai, Phitsanulok	23	10
4. Baan Huay Makang, Tumbol Pha Dad, Amphur Saruay, Chiang Rai	17	18
5. Tumbol Mae Phlu Luang, Amphur Wiang Pha Pao, Chiang Rai	-	6
6. Location between Amphur Wiang Pha Pao to Amphur Prao, Chiang Mai	8	12
7. Baan Pha Bong Ngam, Tumbol Muang Na, Amphur Chiang Dao, Chiang Mai	6	16
8. Tumbol Sob Pong, Amphur Pang Ma Pha, Mae Hong Son	15	14
9. Amphur Ngaw, Lampang	10	7
Northeastern part		
10. Baan Keang, Tumbol Rong Jik, Amphur Phu Ruoe, Loei	24	4
Southern part		
11. Baan Huay Num Kheuw, Tumbol Ron Thong, Amphur Bang Saphan, Prachuap Khiri Khan	15	4
12. Tumbol Thummarat, Amphur Bang Saphan, Prachuap Khiri Khan	13	9
Total	193	128

1.4 Preparation of plant extracts and partially purified fractions.

1.4.1 Preparation of plant extracts.

Thirty-one grounded samples of dried medicinal plants were extracts with ethanol 95 % for 24 hours, thereafter it was filtered and evaporated with rotary evaporator until dried. The amounts of crude extracts remained after evaporation was recorded (Table 5). The highest amount of crude extracts was obtained from Som khaek; (*Garcenia atroviridis* Griff. 3,076.6 mg) Kaan phluu; Clove; (*Eugenia aromaticum* (Linn.) Merr & Perry. 2,911.6 mg) Pooi kak; (*Illicium verum* Hook. 2,656.8 mg) Phluu; Betal ; (*Piper betal* L. 2,334.4 mg) Kek-huai; (*Chrysanthemum indicum* Linn. 2,312.4 mg) and Phet sangkhaat; (*Cissus quadrangularis* Linn. 2,047.2 mg), respectively.

Table 5 Dry weight (mg) of crude extracts per 10 gm dry weight of Thai medicinal plants extracted with 95 % ethanol. (The sequence of Thai medicinal plants are arrange in obtained weight of crude extract.)

Thai medicinal plants				Crude extracts
No.	Thai names	Common names	Scientific names	(mg)
1	Som khaek	-	*Garcenia atroviridis* Griff.	3076.6
2	Kaan phluu	Clove	*Eugenia aromaticum* Linn. Merr & Perry.	2911.6
3	Pooi kak	-	*Illicium verum* Hook.	2656.8
4	Op choei	Cinnamon tree	*Cinnamomum zeylanicum* Linn.	2358.0
5	Phluu	Betal	*Piper betal* L.	2334.4
6	Kek-huai	-	*Chrysanthemum indicum* Linn.	2312.4
7	Phet sangkhaat	-	*Cissus quadrangularis* Linn.	2047.2
8	Bua bok	Asiatic Pennywort	*Centella asiatica* L. Urban.	1930.6
9	Prayong	-	*Aglaia odorata* Lour.	1857.8
10	Kaeo	China Box Tree	*Murraya paniculata* linn. Jack.	1734.1
11	Matuum	Bael Fruit Tree	*Aegle marmelos* L. Corr.	1623.6
12	Ta khrai	Lemon Grass	*Cymbopogon citratus* Stapf	1376.2
13	Krachiap daeng	Roselle	*Hibiscus sabdariffa* Linn.	1354.8
14	Fa thalaaai	-	*Andrographis paniculata* Burm. Wall. Ex. Nees.	1111.4
15	Kham foi	Safflower	*Carthamus tinctorius* Linn.	1025.2
16	Mara khee nok	Bitter Cucumber	*Momordica charantia* Linn.	1020.6
17	Yaa nuat maeo	-	*Orthosiphon grandiflorus* Bolding.	1007.6
18	Khamin chan	Turmeric	*Curcuma domastica* Val.	964.6

Table 5 (Continued)

Thai medicinal plants			Crude extracts
No. Thai names	Common names	Scientific names	(mg)
19 Hanumaan prasaan khai	-	*Scheffera sp.*	937.4
20 Yaa khaa	-	*Imperata cylindrica* Beauv.	854.7
21 Khluu	Indian Marsh Fleabane	*Pluchea indica* Less.	837.4
22 Luuk tai bai	-	*Phyllanthus virgatus* Forst.	800.0
23 Mon	Mulberry tree	*Morus alba* Linn.	689.6
24 Khing	Ginger	*Zingiber officinale* Roscoe	687.2
25 Kheelek	Thai Copper Pod	*Cassia siamea* Lamk	655.6
26 Boraphet	-	*Tinospora crispa* Miers	602.6
27 Yaa haeo moo	Nut Grass	*Cyperus rotundus* Linn.	500.1
28 Yaa dok khaao	-	*Vernonia cinerea* Less.	464.6
29 Chumhet thet	Candelabra Bush	*Cassia alata* Linn.	408.0
30 Krathiam	Garlic	*Allium sativum* Linn.	281.0
31 Raang chute	-	*Thunbergia laurifolia* Linn.	248.2

1.4.2 Partially purification compounds.

There are two medicinal plant extracts; betal vine leave (*Piper betal* L.) and clove flower (*Syzygium aromaticum* (Linn.) Merr & Perry.) from 31 medicinal plant extracts were actively showed inhibitory effects on vegetative growth of pathogens, *F. oxysporum* (See Table 8). These two plant extracts were partially purified by solvent partition technique (Wangkiat, 1995). The table 6 show amount of compound in each organic solvent fraction in dry weight.

Table 6 Amounts of compounds in organic solvent fractions, petroleum ether and ethyl acetate, of betal vine leave and clove crude extracts by using solvent partition technique (Wangkiat, 1995).

Medicinal plant	Fractions	Dry Weight (mg.)
Kaan phluu; Clove flower (*Syzygium aromaticum*)	Petroleum Ether	451.0
	Aqueous I	3785.3
	Ethyl acetate	513.0
	Aqueous II	193.3
Phluu; Betal leave (*Piper betal*)	Petroleum Ether	51.2
	Aqueous I	152.1
	Ethyl acetate	261.4
	Aqueous II	96.8

2. Screening for antagonists and the efficacy test of plant extracts.

2.1 *In vitro* testing for antagonistic ability.

One hundred ninety-three isolates of fungi were tested for antagonistic ability to *F. oxysporum* by using spot inoculation technique (Figure 3). There was twenty-nine isolates showed antagonistic interaction in parasitism, they were rapidly growth or overgrowth the pathogen (*F. oxysporum*). Four isolates showed antagonistic interaction in competitive for space or food, the colonies of antagonists and pathogens were grown against each other. Fourteen isolates showed antagonistic interaction in antibiosis, they provided clear zone surrounding the antagonistic colonies. (Table 7)

In case of antagonistic bacteria, one hundred twenty-eight isolates were investigated for antagonistic ability by using filter paper disc method (Figure 4). Nine isolates showed competitive interaction while 8 isolates showed antibiosis interaction (Table 7).

Table 7 Antagonistic interaction of microorganism against ginger rhizome rot pathogen, *Fusarium oxysporum*, under laboratory condition.

Antagonistic Microorganism	Interaction (isolate)			Total (isolate)
	Competitive	Parasitism	antibiosis	
Fungi[1/]	4	29	14	47
Bacteria[2/]	9	-	8	17
Total	13	29	22	64

[1/] Spot inoculation (Inwang and Chamswang, 1986)

[2/] Filter paper disc method (Dhingra and James, 1995)

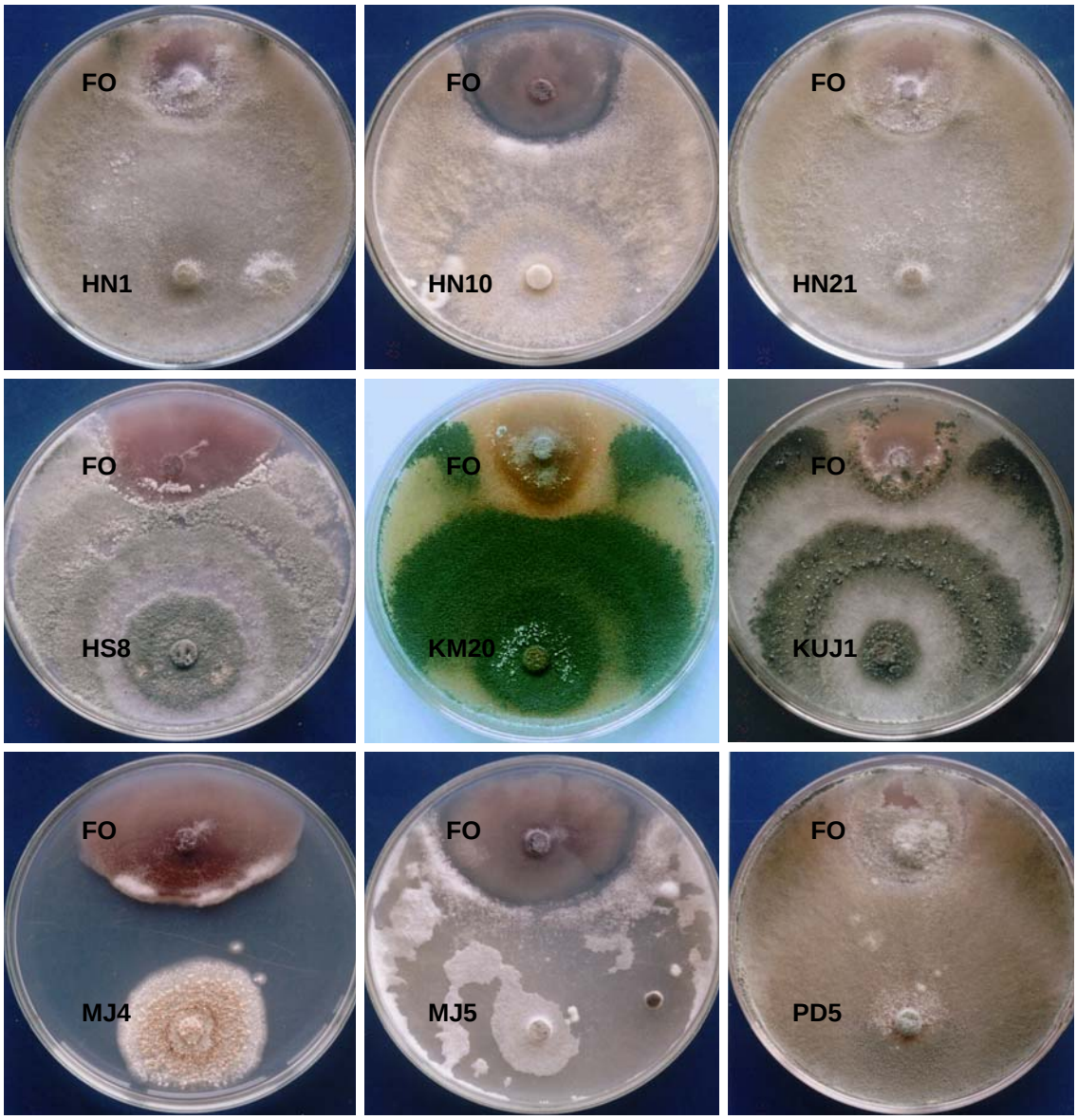

Figure 3 Antagonistic interaction of antagonistic fungi (lower colonies in Petri dish) and the pathogen (FO: *Fusarium oxysporum*) by using spot inoculation test (Inwang and Chamswang, 1986). Two isolates namely KM20 and PD5 was selected for further study against storage rot of ginger.

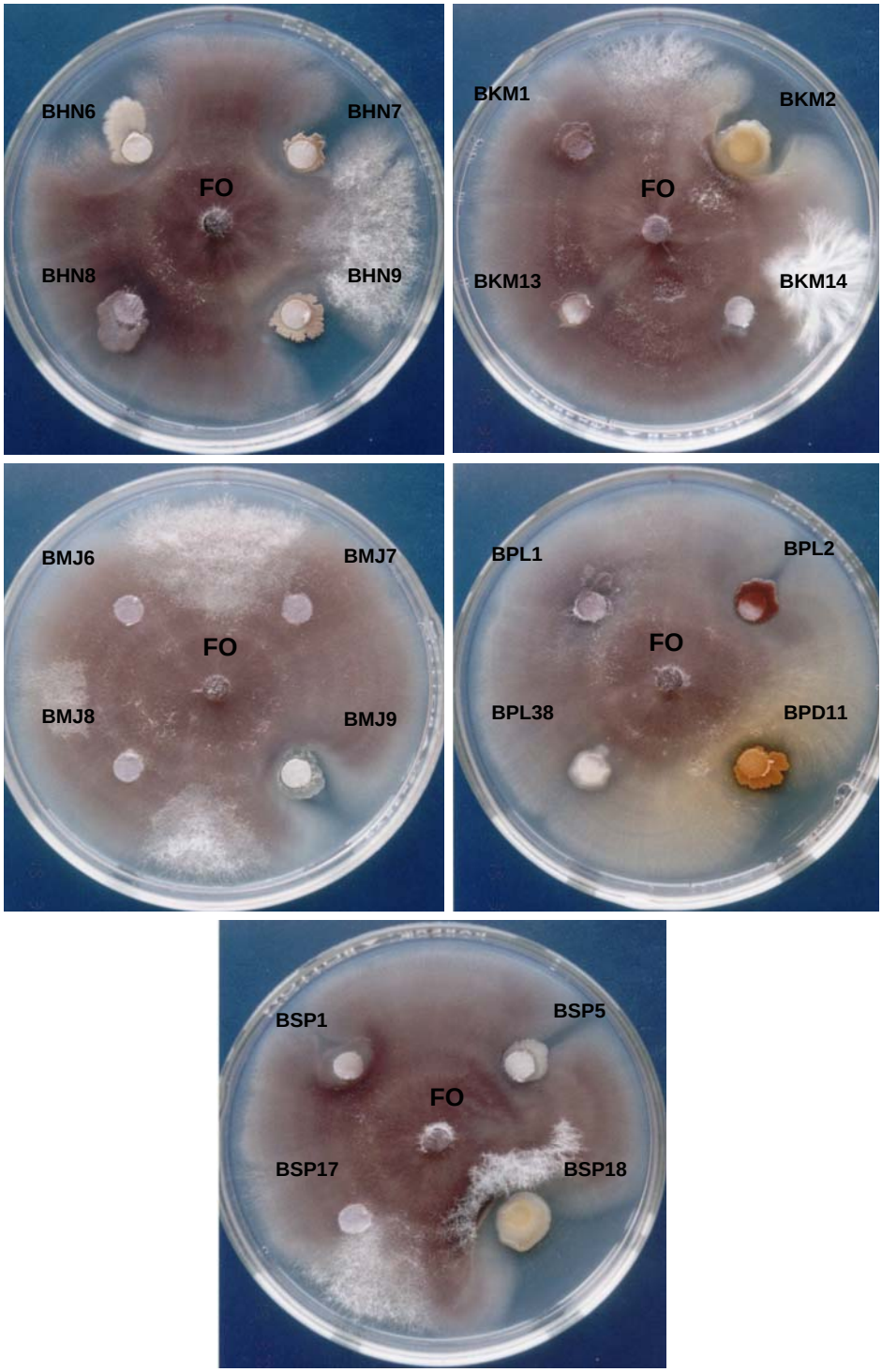

Figure 4 The interaction of antagonistic bacteria (outer colonies in Petri dish) and the pathogen (FO: *Fusarium oxysporum*) by using filter paper disc method (Dingra and James, 1995).

2.2 Testing for growth of antagonists on ginger rhizomes.

Two isolates of selected antagonistic fungi ‘KM20’ and ‘PD5’ were tested for growth on ginger rhizomes. Those fungi were identified as *Trichoderma sp.*. The population of both isolates of antagonistic fungi on wounded tissue was increased faster than the population of fungi on rhizome skins. The population of fungi on wounded tissue was 2 log of cfu/ml higher than the population of fungi on skins (Figure 5 A, B). Furthermore, the population of antagonistic fungi isolate ‘PD5’ on the skin of rhizome was decreased drastically and could not be detected at 8 DAI.

Firmness of rhizome tissue was measured at both sites of inoculated wound and skin tissues. The results showed that the isolate ‘PD5’ provided the firmness values of wounded tissue better than isolate ‘KM20’, whereas the firmness of skin tissue was not distinctly differences. (Figure 6 A, B)

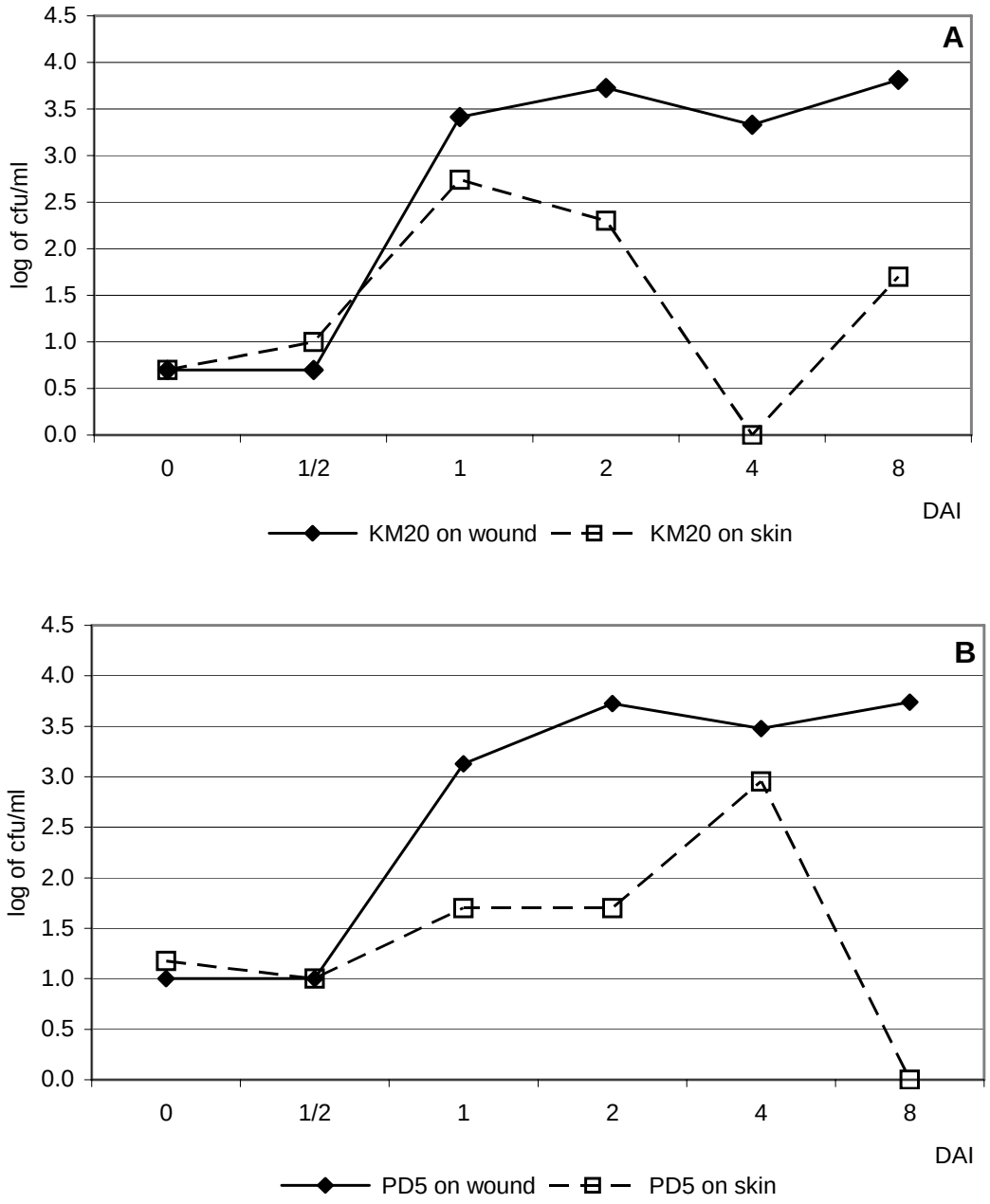

Figure 5 The population changes of antagonistic fungi 'KM20' (A) and 'PD5' (B) on wound and skins tissue, 8 days after inoculation (DAI).

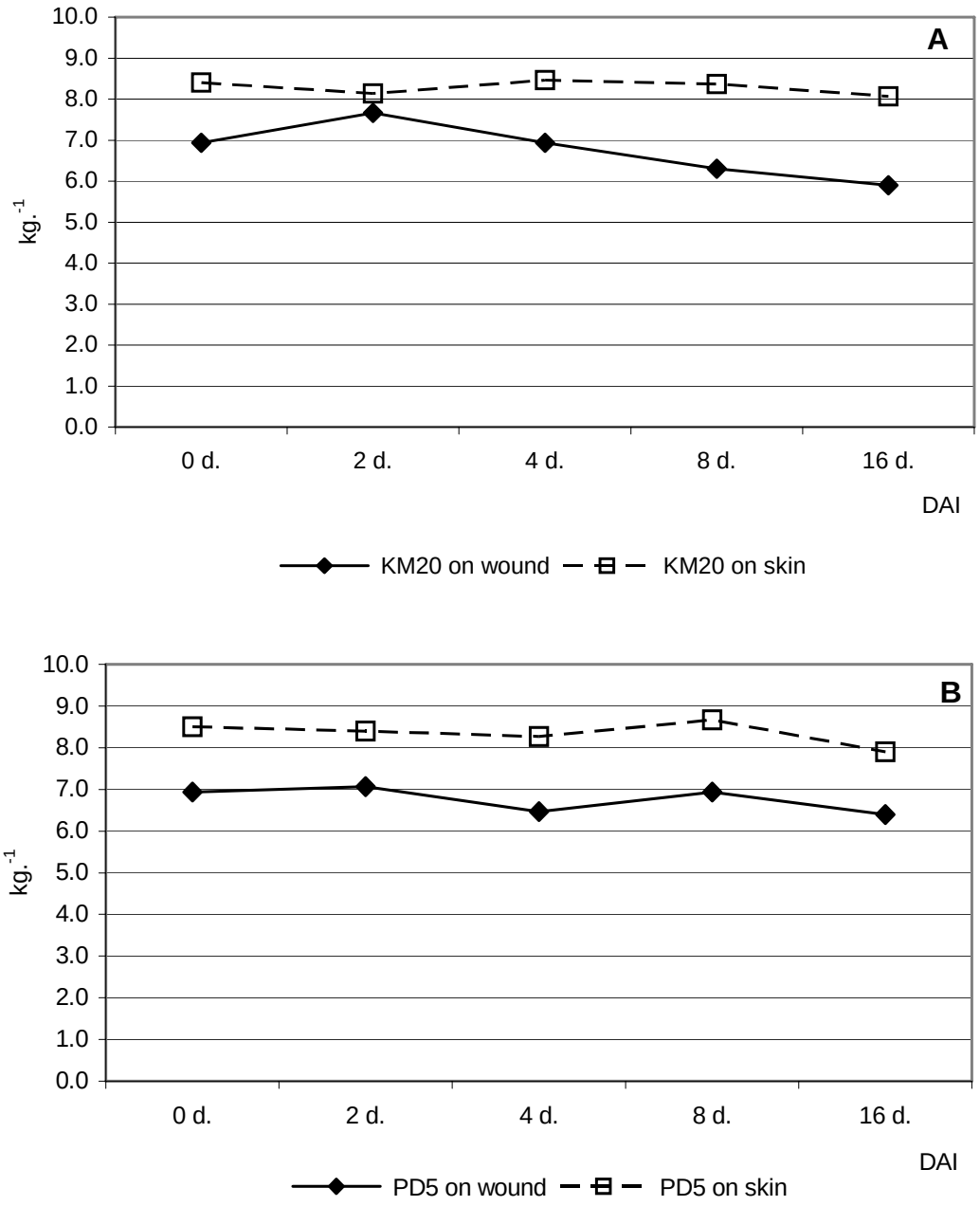

Figure 6 Firmness of tissue changes at wound and skins tissue of ginger rhizome after inoculation with antagonistic fungi 'KM20' (A) and 'PD5'(B), 0-16 days after inoculation (DAI).

2.3 Testing for the efficacy of culture filtrate of antagonistic microorganism on vegetative growth of pathogen.

The result showed that those culture filtrates of antagonistic *Trichoderma* isolate 'KM20' and 'PD5' and antagonistic bacteria isolate 'BMJ7' and 'BPL2' were not provided clear zone on vegetative growth of *F. oxysporum*. (Figure 7)

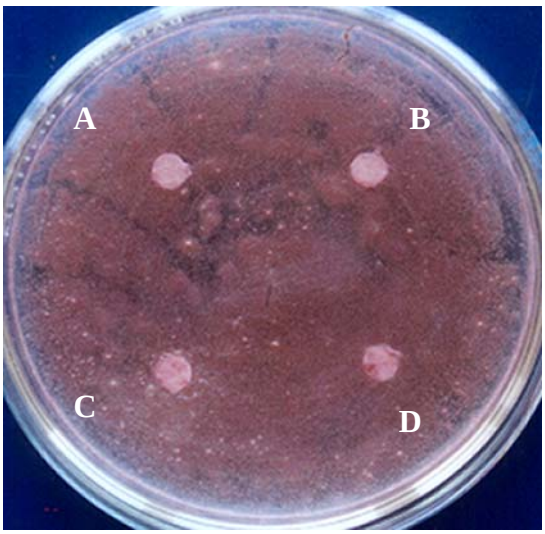

Figure 7 The culture filtrates of antagonistic *Trichoderma* isolate 'KM20' (A), 'PD5' (B) and antagonistic bacteria isolate 'BMJ7' (C), 'BPL2' (D) were not showed clear zone on vegetative growth of *Fusarium oxysporum*.

2.4 Testing for the efficacy of plant extracts on vegetative growth of pathogens.

2.4.1 Testing for inhibitory effect of plant extracts on vegetative growth of the pathogens.

Inhibitory effect of plant extracts on *F. oxysporum* was tested by Filter paper disc method in laboratory. It revealed that the extract of betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) had inhibitory effect on vegetative growth of *F. oxysporum*. The inhibitory reaction was observed by measuring clear zone surrounding the paper disc (Figure 8; Table 8). The diameter of clear zone was differed between plant extracts and their concentrations. The extract of betal vine leave (*Piper betal* L.) provided the largest clear zone at concentration 100,000 ppm (2.45 cm.). It was 78.27 % of clear zone diameter of the fungicide, 'Imazalil' 1,000 ppm (3.13 cm.) which used as positive check. Whereas, the extract of clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) at 100,000 ppm was expressed 65.49 % of the diameter of clear zone (2.05 cm.) compared to 1,000 ppm Imazalil.

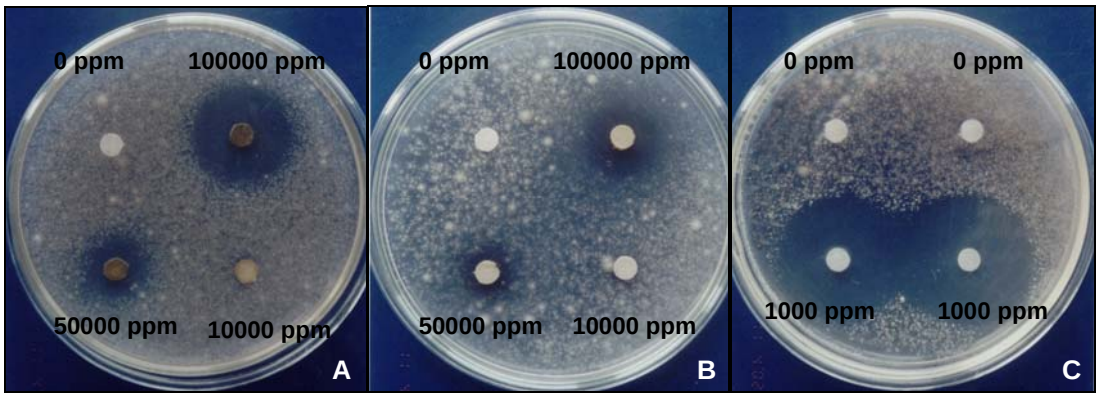

Figure 8 Clear zone expression from inhibitory effect of the extract of (A) betal vine leave (*Piper betal* L.) and (B) clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) on vegetative growth of *F. oxysporum* concentrations at 0, 10,000, 50,000 and 100,000 ppm and (C) Imazalil at concentration 1,000 ppm.

Table 8 Diameter of clear zone proceeds from inhibitory effect of the extract of betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) on vegetative growth of *F. oxysporum* at various concentrations compared to Imazalil.

Plant extracts	conc.(ppm)	Diameter of clear zone (cm.)[1]
betal vine leave	10,000	0
	50,000	1.25
	100,000	2.45
clove	10,000	0
	50,000	1.00
	100,000	2.05
Imazalil	1,000	3.13
Control		0

[1] Average from 3 replications

2.4.2 The efficacy of partially purified plant extracts.

The efficacy of partially purified of betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) extracts by using solvent partitioning technique on growth inhibition of *F. oxysporum* was carried out in laboratory. The results showed that the aqueous1 fraction of betal vine leave extract provided the largest clear zone at concentration 100,000 ppm (36.21 % compared to Imazalil 1000 ppm, positive check), whereas petroleum ether fraction of clove extract provided the largest clear zone 32.67 % at concentration 100,00 ppm (Table 9).

Table 9 Percentage of clear zone diameter of partially purified fractions of betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) at various concentrations on inhibition of vegetative growth of *Fusarium oxysporum* compared to 1,000 ppm Imazalil.

Herb	Fractions	Concentrations (ppm)		
		100,000	50,000	10,000
Clove	Petoleum ether	32.76	18.97	5.17
	Aqueous1	10.34[1]	3.45	3.45
	Ethylacetate	0.00	0.00	0.00
	Aqueous2	0.00	0.00	0.00
Piper betal	Petoleum ether	0.00	0.00	0.00
	Aqueous1	36.21	0.00	0.00
	Ethylacetate	29.31	10.34	0.00
	Aqueous2	0.00	0.00	0.00
Negative check (Ethanol)		0.00	0.00	0.00
Positive check (Imazalil 1,000 ppm)		100.00	100.00	100.00

[1]Average from 3 replications

2.5 The efficacy of antagonists and plant extracts on controlling ginger rhizome rot (*Fusarium* rot) during storage.

2.5.1 The efficacy of antagonists on controlling of ginger rhizome rot.

Two isolates of antagonists were tested to control ginger rhizome rot during storage. The results showed that the populations of antagonistic fungi isolates 'KM20' and 'PD5' on wounded tissues was increased regularly after ½ DAI until the end of experiment (Figure 9 A), whereas the increasing of population on skins was limited at 1 DAI and consistent in change until the end of experiment (Figure 9 B). In case of *F. oxysporum* population change on ginger rhizome, it was found that the change was the same tendency as those antagonistic fungi on both wounded and skin of rhizome.

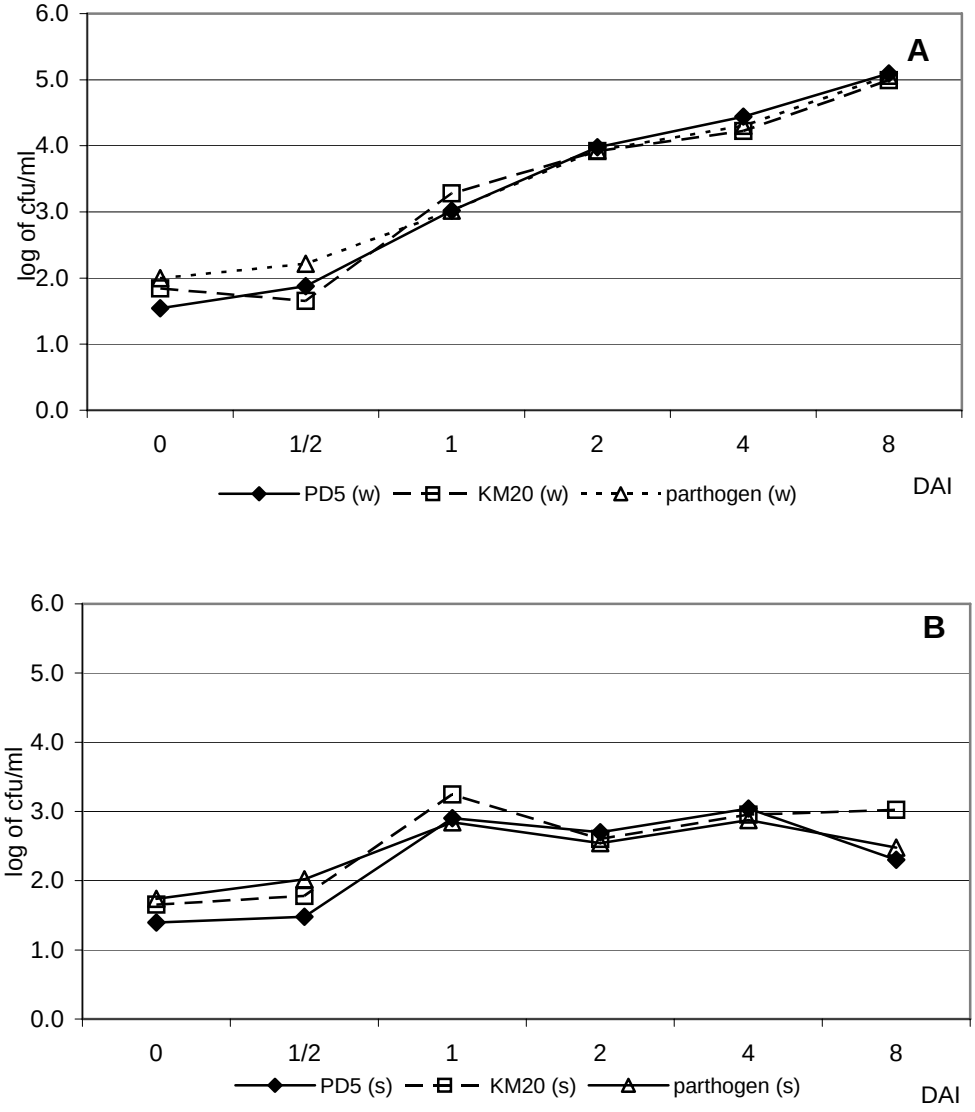

Figure 9 The population changes of antagonistic fungi isolates 'KM20' and 'PD5' and the pathogen *F. oxysporum* on wounded tissues (A) and skins (B) of ginger rhizome.

2.5.2 The efficacy of plant extracts on controlling ginger rhizome rot.

The efficacy of betal vine leave extracts was tested on population change of *F. oxysporum* at wounded tissues was not-detected at 0 – 1 DAI and then slightly increased at 2 DAI until 8 DAI, whereas the population change in clove extract treatment was decreased at 0 – ½ DAI and then sharply increased at 1 to 8 DAI (Figure 10 A). On skin tissue, the population of *F. oxysporum* in clove and betal vine leave extracts treatments were fluctuated changes between 0 – 3.0 log of cfu/ml (Figure 10 B). However, the population of *F. oxysporum* on both wound and skin tissues of ginger rhizome treated with 1,000 ppm fungicide Imazalil were lower than another treatments.

The firmness of wounded tissue was slightly decreased in ginger rhizome treated with clove and betal vine leave extracts at 2 DAI, while another treatments were steadily change (Figure 11 A). The firmness on skin tissue of ginger treated with in clove extract was decreased, whereas betal vine leave extract, non-treated and only *F. oxysporum* inoculation treatments were increased (Figure 11 B).

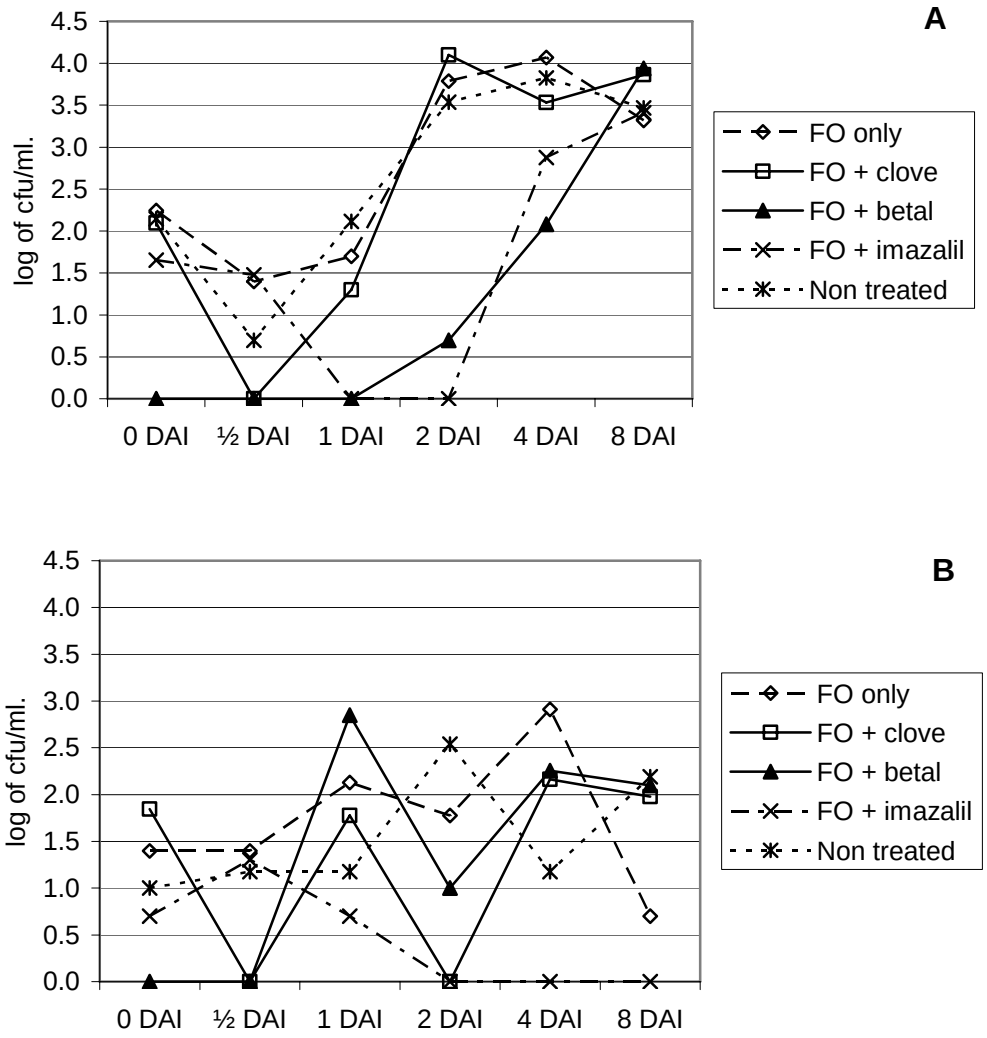

Figure 10 The population changes of *Fusarium oxysporum* on wounded tissue (A) and skin tissue (B) of ginger rhizome treated with 10,000 ppm of clove and betal vine leave extracts compared to 1,000 ppm of Imazalil at 0, ½, 1, 2, 4 and 8 DAI (Days After Inoculation).

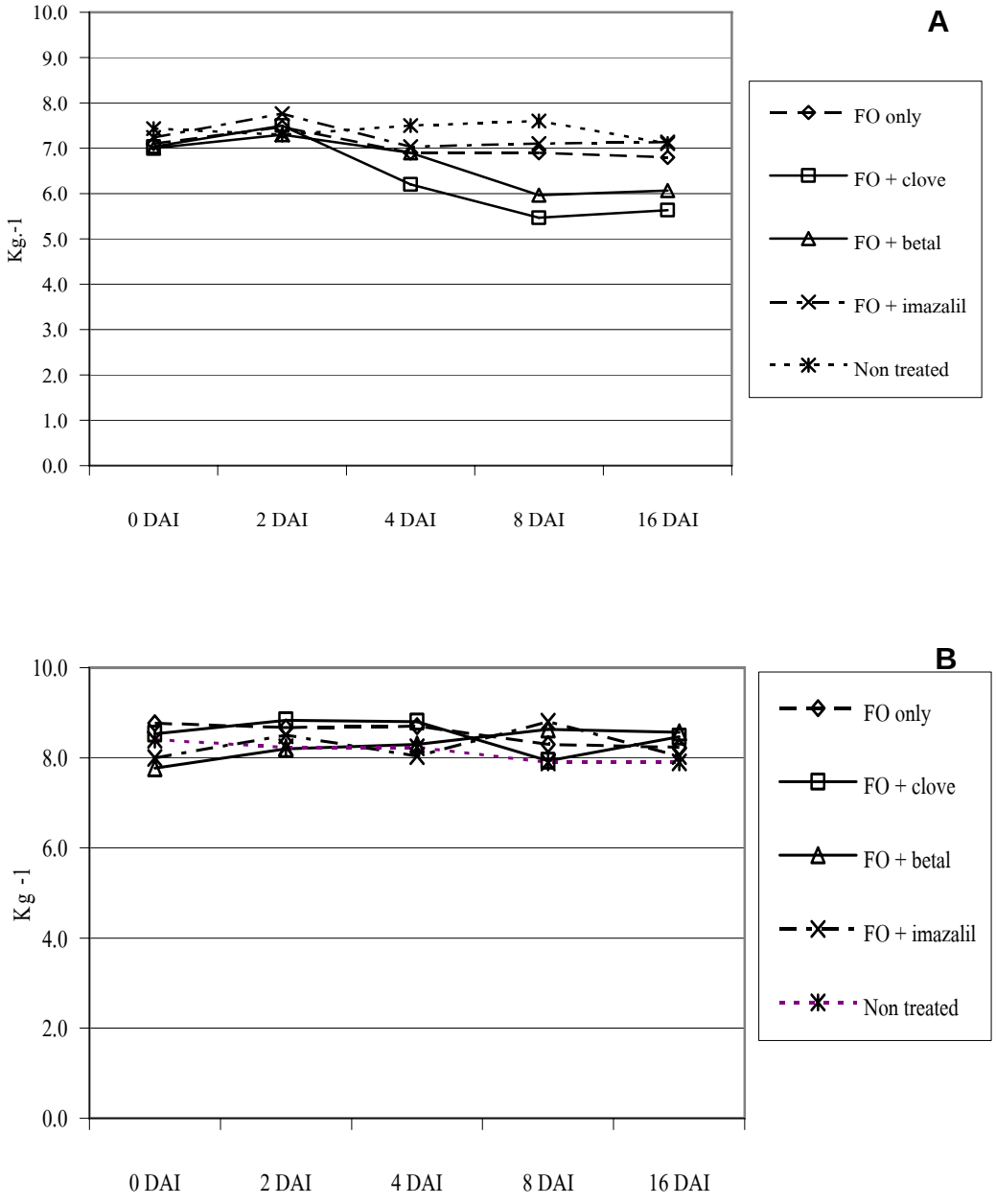

Figure 11 The firmness changes of wounded tissue (A) and skin tissue (B) of ginger rhizome treated with 10,000 ppm of clove and betal vine leave extracts compared to 1,000 ppm of Imazalil at 0, ½, 1, 2, 4, 8 and 16 DAI (Days After Inoculation) with *Fusarium oxysporum*.

3. The examination of some compounds mixed up with plant extract and antagonistic microorganism to control ginger rhizome rot.

3.1 *In vitro* screening of some compounds on vegetative growth of the pathogen (Figure 12).

The mycelium of *F. oxysporum* was grown cover the surface of PDA medium plus D-fructose in the Petri dish at 10 DAI (Figure 12 A). The PDA plus D-fructose medium provided percentage of growth inhibition at concentration 0.5, 1.0, 2.0 and 4.0 % were -6.67, -6.00, -4.67 and -1.00 %, respectively.

The percentage of growth inhibition of PDA plus $CaCl_2$ medium on *F. oxysporum* at concentration 0.5, 1.0, 2.0 and 4.0 % were -3.67, -1.00, 14.33 and -66.67 %, respectively. (Figure 13 A, Table 10)

Chitosan plus PDA medium provided the percentage of growth inhibition at concentration 0.1, 0.2, 0.4 and 0.6 % were 37.00, 43.67, 61.67 and 75.33 %, respectively (Figure 13 B, Table 10), whereas, the positive control, Imazalil provided 100 % of growth inhibition at concentration 0.1 % (1,000 ppm) (Figure 12 D2, Table 10).

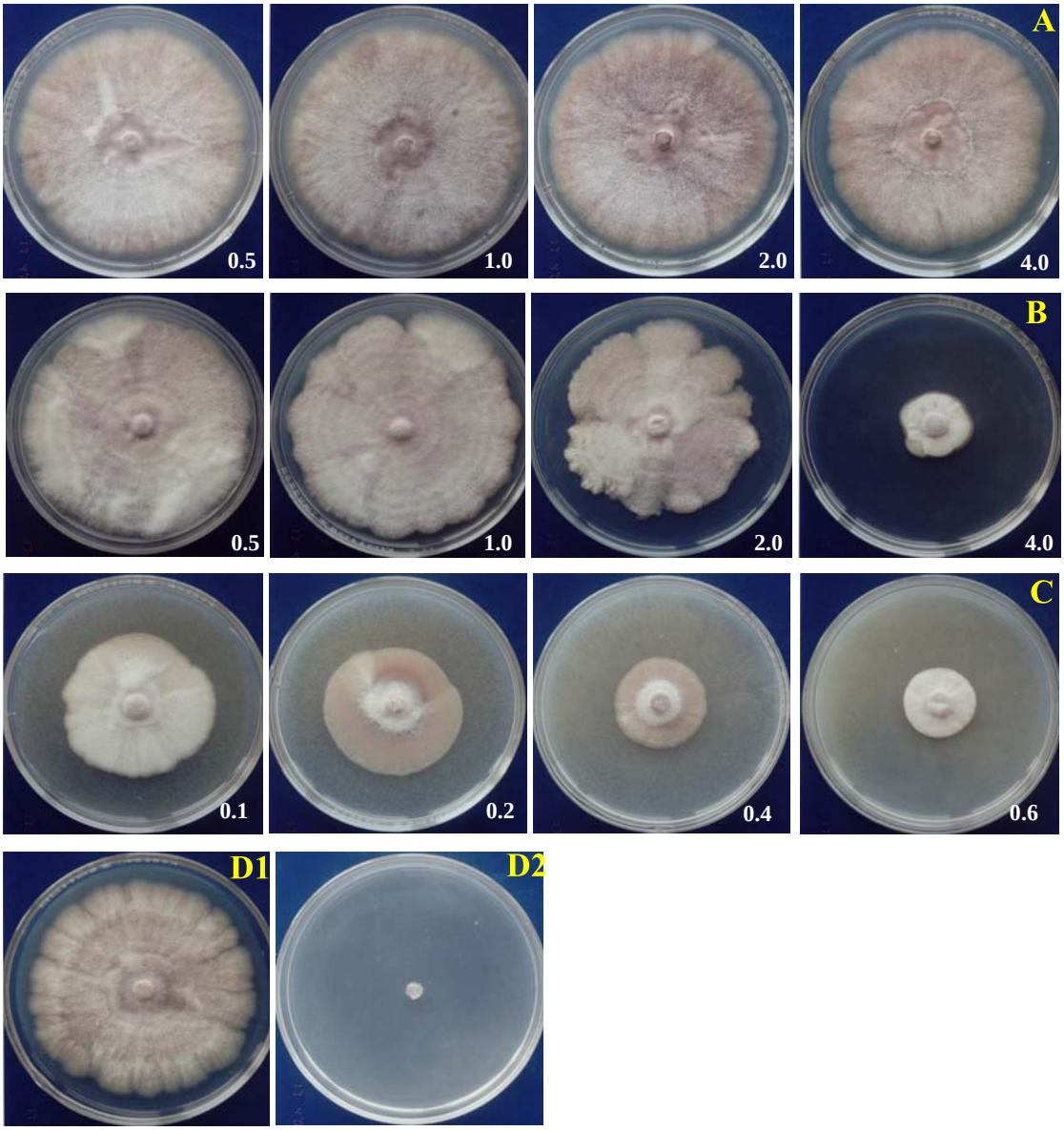

Figure 12 Effects of some compounds plus PDA medium on mycelium growth of *Fusarium oxysporum*.

A : D-fructose 0.5, 1.0, 2.0 and 4.0 %
B : $Cacl_2$ 0.5, 1.0, 2.0 and 4.0 %
C : Chitosan 0.1, 0.2, 0.4 and 0.6 %
D1 : Control
D2 : Imazalil 0.1 % (1,000 ppm)

Table 10 The percentage of growth inhibition of some compound plus PDA D-fructose, $CaCl_2$, Chitosan and Imazalil on mycelium growth of *Fusarium oxysporum*.

Treatment	Concentration	growth inhibition[1] (%)
Imazalil	0.1 % (1000 ppm)	100.00[2]
D-fructose	0.5 %	-6.67
	1.0 %	-6.00
	2.0 %	-4.67
	4.0 %	-1.00
$CaCl_2$	0.5 %	-3.67
	1.0 %	-1.00
	2.0 %	14.33
	4.0 %	66.67
Chitosan	0 %	22.00
	0.1 %	37.00
	0.2 %	43.67
	0.3 %	60.33
	0.4 %	61.67
	0.6 %	75.33
Control (PDA plus steriled water)		0

[1] Calculation

$$\% \text{ growth inhibition} = \frac{A - B}{A} \times 100$$

A = colony diameter of control – 0.6 (cm.)

B = colony diameter of treatment – 0.6 (cm.)

[2]Average from 3 replications

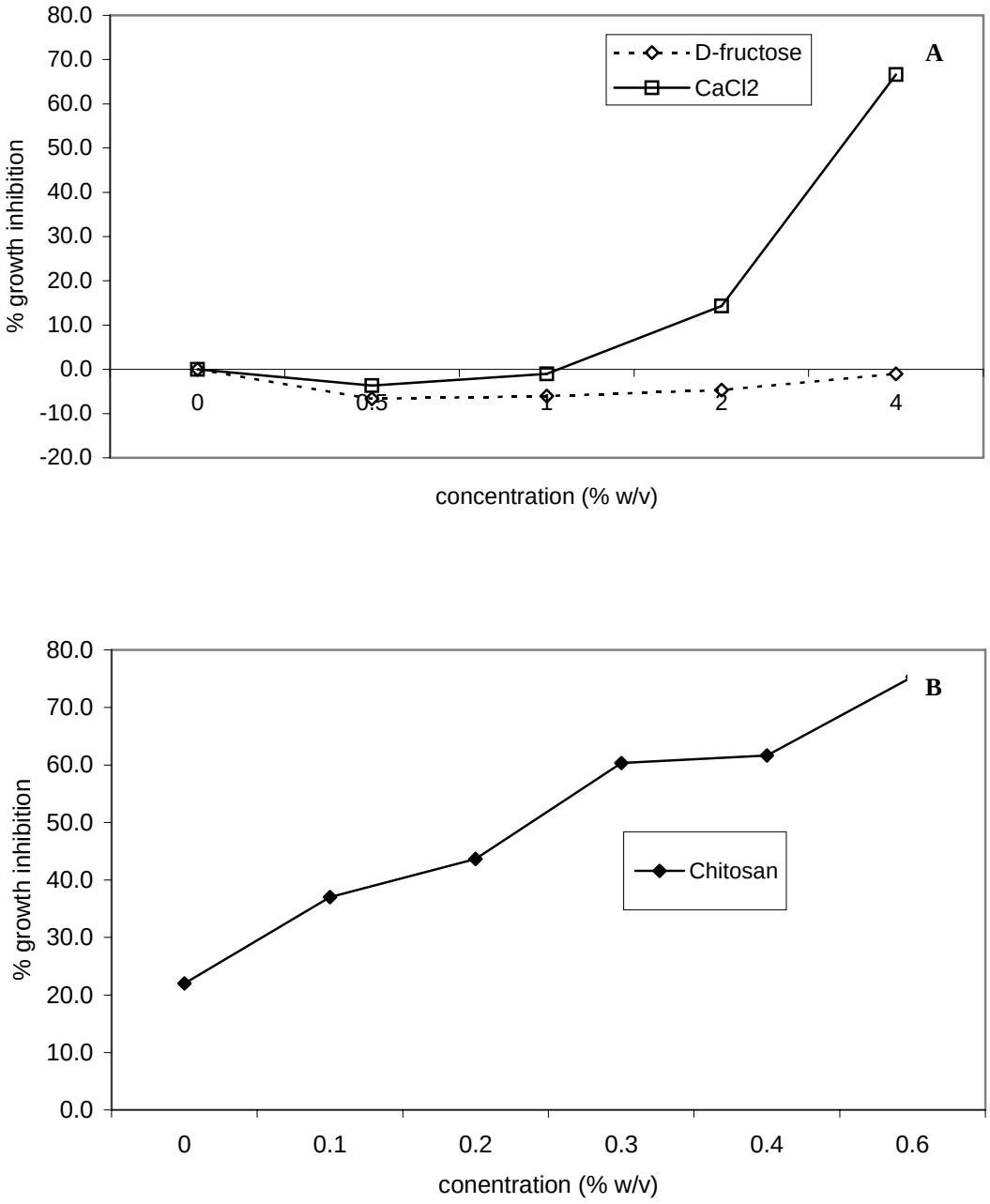

Figure 13 The percentage of growth inhibition of some compound plus PDA medium on mycelium growth of *Fusarium oxysporum*.

A = D-fructose and $CaCl_2$

B = Chitosan

3.2 *In vitro* testing of some compounds and plant extracts on growth of antagonistic microorganism.

3.2.1 Effect of plant extract on microorganisms.

Effect of clove and betal vine leave extracts were tested on vegetative growth of antagonistic fungi at 100,000 and 50,000 ppm by using filter paper disc method. The results showed that fungal isolate 'MJ4' provided the tolerance to 100,000 ppm of the both betal vine leave and clove extracts at index 4 (highest tolerance) and isolates 'SP4, RT1, RJ6, PD8, PD2, PD12, KUJ6, KM20, HN2 and HN11' provided the tolerance to 100,000 ppm of clove extracts at index 4. (Figure 14 A, B)

Antagonistic bacteria tolerated to clove and betal vine leave extracts at the concentration 100,000 and 50,000 ppm were conducted by using filter paper disc method. The smaller diameter of clear zone, which produced by inhibitory effect of extract on bacterial growth indicated to the tolerance of bacteria. The results showed that those bacterial isolates 'BKM10, BKM8 BPL1 and BPL2' provided the smallest clear zone at 100,000 ppm of betal vine leave extracts, while the isolates 'BKM10 and BPL1' provided smallest clear zone at 100,000 ppm of clove extracts (Figure 15 A, B)

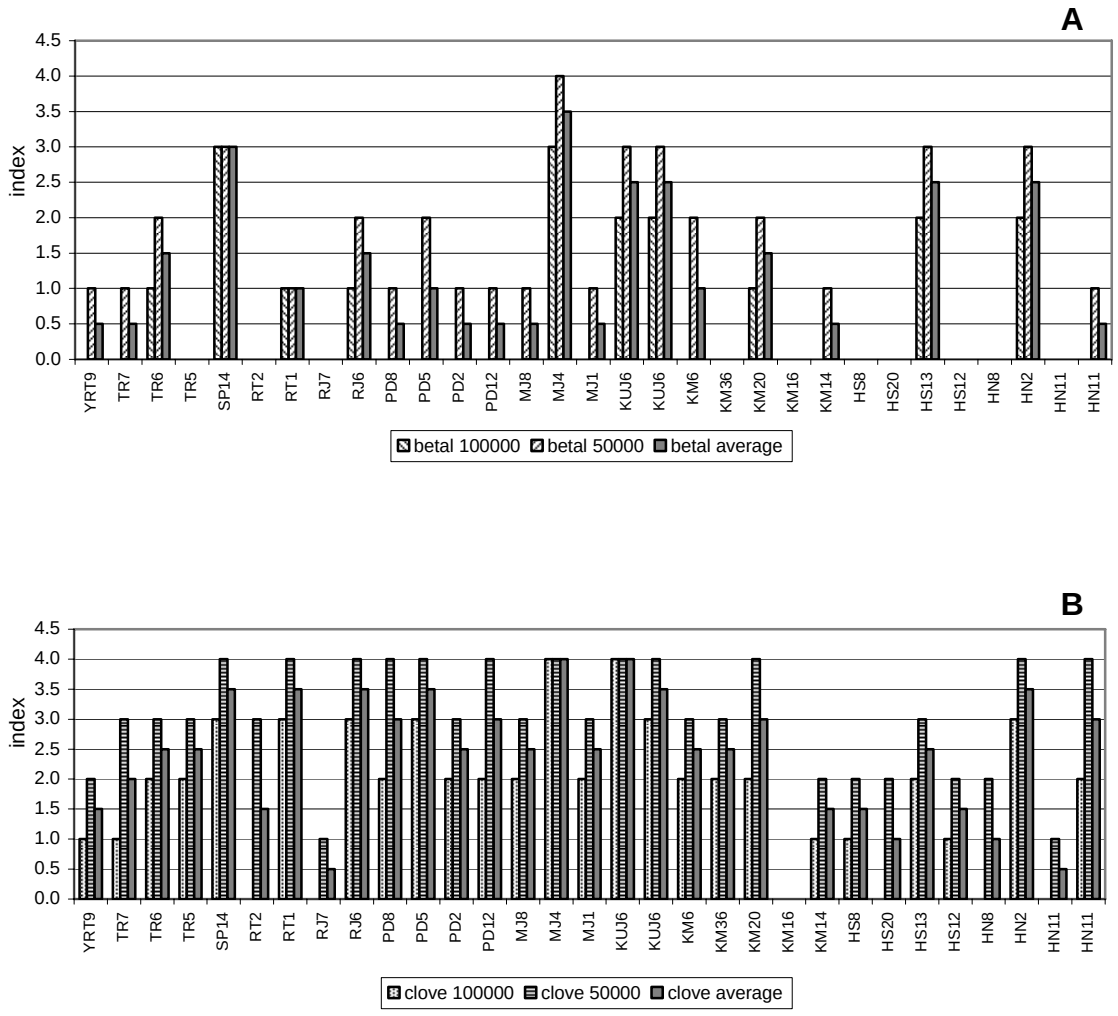

Figure 14 The tolerant index of antagonistic fungi to betal vine leave (A) and clove (B) extracts at concentration 100,000 and 50,000 ppm.

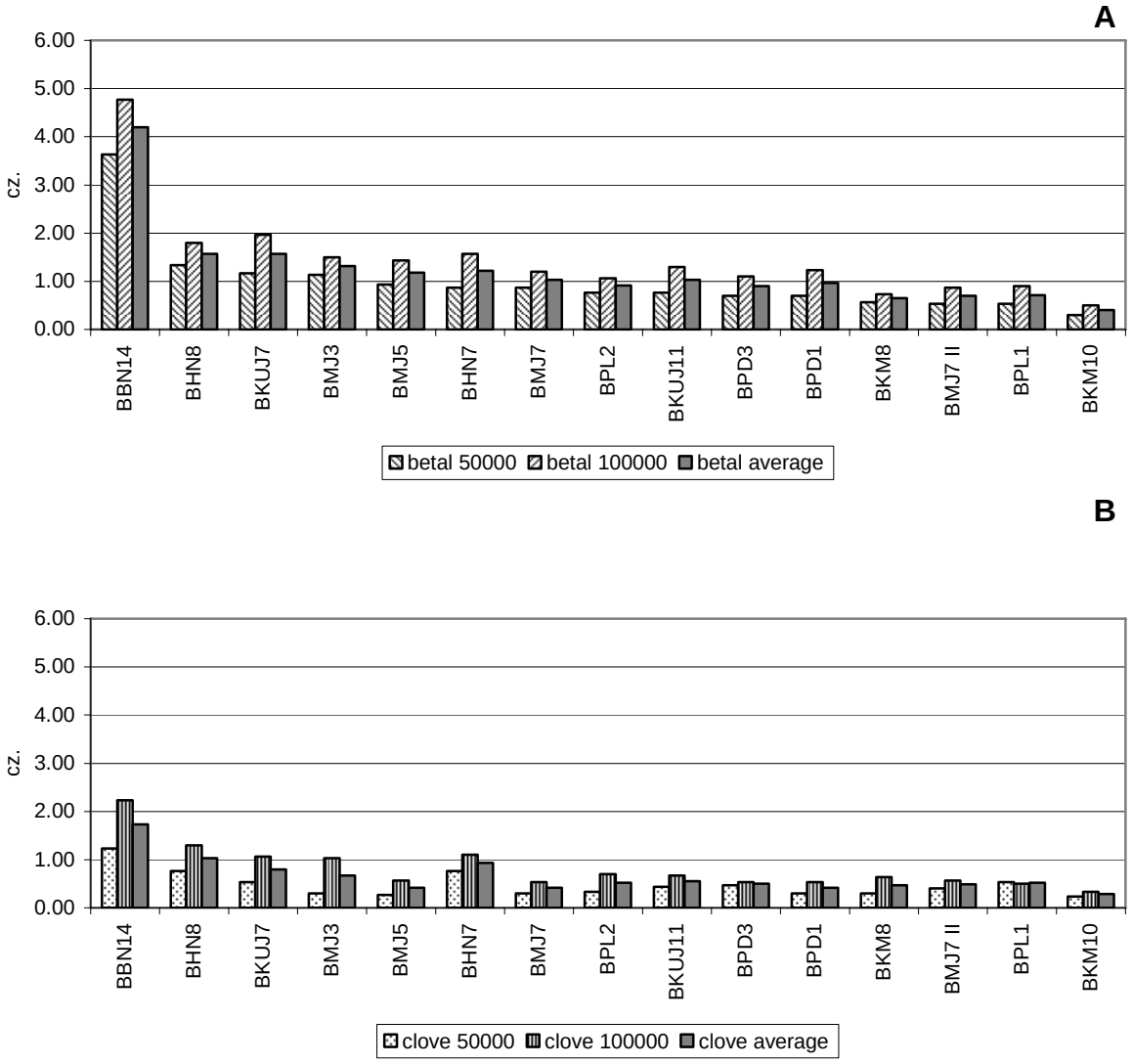

Figure 15 The clear zone produced by inhibition effect of antagonistic bacteria to betal vine leave (A) and clove (B) extracts at concentration 100,000 and 50,000 ppm.

3.2.2 Effect of some compounds on microorganisms.

Effect of compounds D-fructose, Chitosan and $CaCl_2$ on vegetative growth inhibition of antagonistic fungi were conducted by using poison food technique. The colonies diameters of tested fungi were calculated to percentage of growth inhibition from control treatment. The results showed that those fungal isolates, 'KM20 and PD5' were provided highest growth rate (3 DAT) and can be growth promoted by 2 % of D-fructose and $CaCl_2$, whereas RT1 was promoted by D-fructose and $CaCl_2$ at 2 % and 4 % (Table 11, Figure 16).

Effect of compounds D-fructose, Chitosan and $CaCl_2$ on growth inhibition of antagonistic bacteria were conducted by using poison food technique. The colonies diameter of bacteria was calculated to % growth inhibition. The results showed that only 'BPL2' could be growth promoted by 4 % of D-fructose and 2 % of $CaCl_2$ at 1 DAT whereas the others were inhibited. Thereafter, all of tested antagonistic bacteria isolates were responsed to the compounds as growth inhibition (Figure 17).

The results revealed that some tested antagonistic fungi and bacteria were not showed growth inhibition by some compounds. In contrast, it promotes the growth of them.

Table 11 Growth inhibition percentage of 8 antagonistic fungi isolates on PDA plus D-fructose, Chitosan and $CaCl_2$, 3-8 days after treatment (DAT).

Isolates of Antagonistic fungi	Compounds						
	D-fructose		chitosan		$CaCl_2$		DAT
	2%	4%	0.3 %	0.6 %	2%	4%	
KM20	-13.33[1]	57.33	94.67	100.00	-13.33	17.33	3
PD5	-46.55	25.86	93.10	96.55	-46.55	-6.90	3
RT1	-254.17	-150.00	37.50	95.83	-254.17	0.00	4
RJ6	21.18	55.29	69.41	85.88	11.76	36.47	4
MJ4	8.24	14.12	25.88	55.29	9.41	11.76	7
SP14	32.94	63.53	0.00	29.41	28.24	52.94	8
HN2	-53.06	-32.65	-73.47	-38.78	-46.94	-42.86	8
HS13	-30.36	3.57	-51.79	-28.57	-14.29	-44.64	8

[1] Average from 3 replications

[2] Calcultion

$$\% \text{ growth inhibition} = \frac{A - B}{A} \times 100$$

A = colony diameter of control

B = colony diameter of treatment

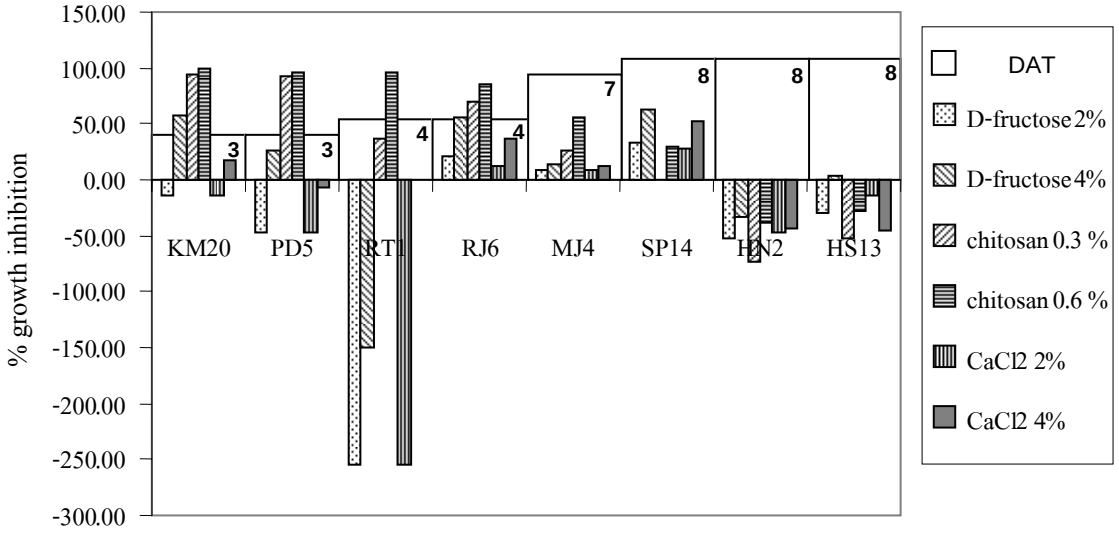

Figure 16 Growth inhibition percentage of antagonistic fungi, which was cultivated on PDA plus D-fructose, Chitosan and $CaCl_2$ at 3-8 days after treatment (DAT).

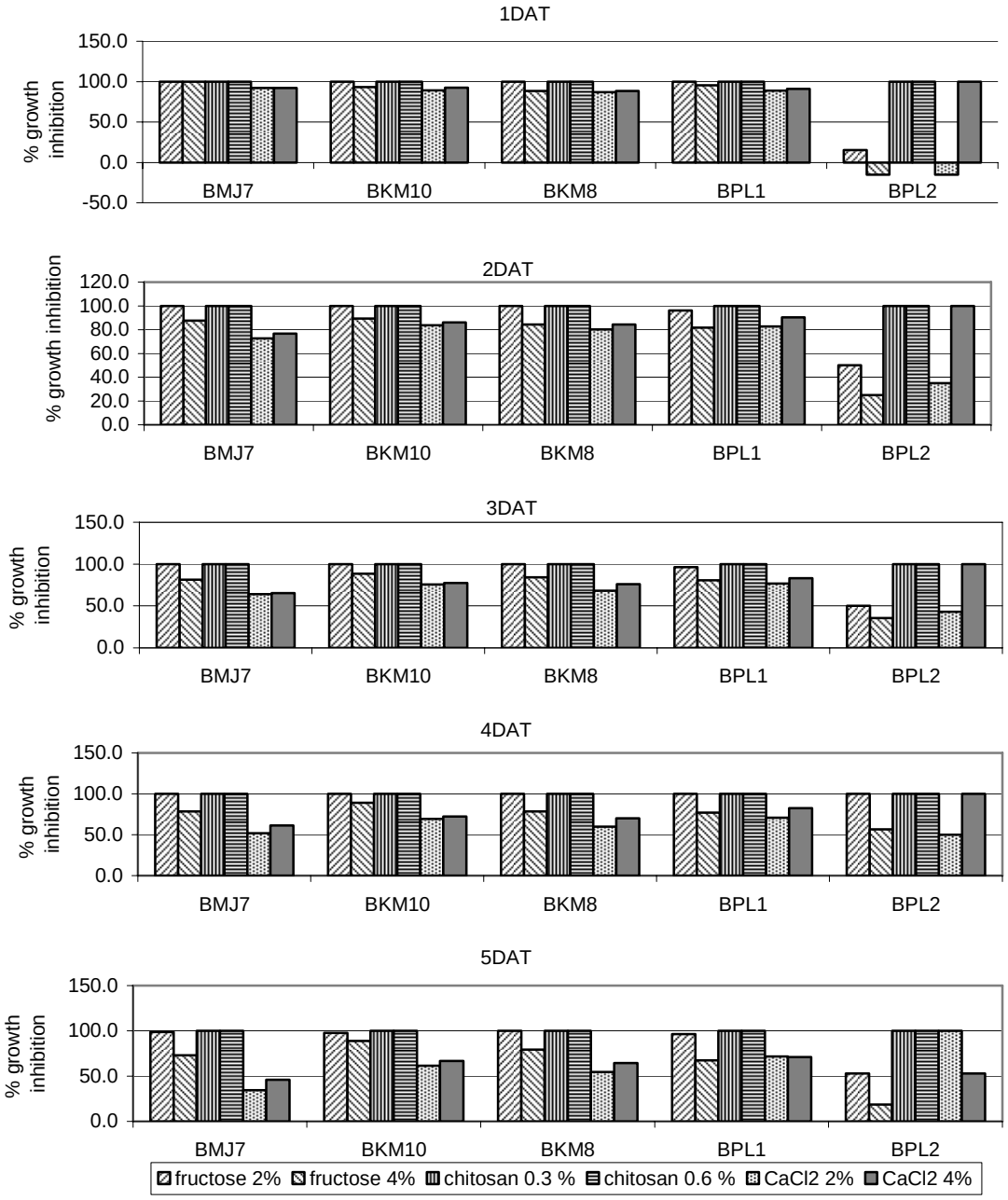

Figure 17 Growth inhibition percentage of antagonistic bacteria, which was cultivated on PDA plus D-fructose, Chitosan and $CaCl_2$ at 3-8 days after treatment (DAT).

3.3 Control of ginger rhizome rot during storage by using antagonistic microorganism with enhancing compounds and plant extracts.

The antagonistic bacteria ‘BPL2’ and fungi ‘KM20’ ‘PD5’with enhancing compounds, Chitosan, $CaCl_2$ and clove and betal vine leave extracts were used in combination for controlling of ginger rhizome rot during storage.

The experiment of ginger rhizome in combination amount compounds (Chitosan, $CaCl_2$) and antagonistic microorganism were conducted to compare with Imazalil fungicide. The acceptable results were shown in treatments of antagonistic bacteria ‘BPL2’, antagonistic fungi ‘PD5’ + clove 2000 ppm, antagonistic fungi ‘PD5’, Clove 2,000 ppm and chitosan 0.3 % with disease index (DI) 6.3, 7.7, 9.3, 9.3 and 9.7, respectively (Figure 18 and Table 12). The treatments those consisting of antagonistic fungi ‘PD5’ were also discarding in order to provide the green color of fungal mycelium on wounded tissue.

The following experiment was conducted by using those selected treatments from the previous experiment. There were chitosan 0.3 %, Clove 2,000 ppm, antagonistic bacteria ‘BPL2’ compared to Imazalil 1,000 ppm. The results showed that antagonistic bacteria ‘BPL2 gave lowest disease index, 6.0, except Imazalil 1,000 ppm, 0.0, (Figure 19 and Table 13) whereas, the others treatments were showed higher disease index.

The semi-commercial experiment was conducted with treatments of Clove 2,000 ppm, antagonistic bacteria ‘BPL2’ compared to Imazalil 1,000 ppm. The results showed that antagonistic bacteria ‘BPL2’ gave the significantly lowest disease index, 4.33 and percentage of rotten rhizome, 8.4 %, except Imazalil 1,000 ppm, 0.03 and 0.0 % respectively (Table 14). While, the clove 2000 ppm and control treatments gave the highest disease index, 7.67 and 8.03, and highest % decay, 49.6 and 48.2 %, respectively.

Table 12 Disease index of ginger rhizome treated with compounds, Chitosan, $CaCl_2$, clove and betal vine leave extracts, antagonistic bacteria 'BPL2' and antagonistic fungi 'KM20' 'PD5' compared to fungicide Imazalil at 1-4 weeks under storage condition (13 °C, 65 % RH).

	Treatments	Disease Index (DI)			
		1st wk.	2nd wk.	3rd wk.	4th wk.
1	Chitosan 0.3 %	4.0[1]	7.3	7.7	9.7
2	$CaCl_2$ 2 %	9.3	9.7	9.7	10.0
3	Clove 2000 ppm	4.0	5.7	7.3	9.3
4	Betal 2,000 ppm	10.0	10.0	10.0	10.0
5	Imazalil 1,000 ppm	1.0	9.0	9.7	9.3
6	BPL2	1.0	1.7	3.0	6.3
7	KM20	10.0	10.0	10.0	10.0
8	PD5	10.0	7.0	8.0	9.3
9	KM20 + CaCl2 2%	10.0	9.7	10.0	10.0
10	KM20 + Betal 2,000 ppm	10.0	9.7	10.0	10.0
11	KM20 + Clove 2,000 ppm	10.0	10.0	10.0	10.0
12	PD5 + CaCl2 2 %	9.7	9.7	10.0	10.0
13	PD5 + Clove 2,000 ppm	9.0	7.0	6.7	7.7
14	Control (DW)	6.7	7.7	8.3	9.3

[1]Average from 3 replications

Note : Disease index (DI) of ginger rhizome rot, mean :

1 = no infection
2 = 10 % of rhizome was infected
3 = 20 % of rhizome was infected
4 = 30 % of rhizome was infected
5 = 40 % of rhizome was infected
6 = 50 % of rhizome was infected
7 = 60 % of rhizome was infected
8 = 70 % of rhizome was infected
9 = 80 % of rhizome was infected
10 = > 90 % of rhizome was infected

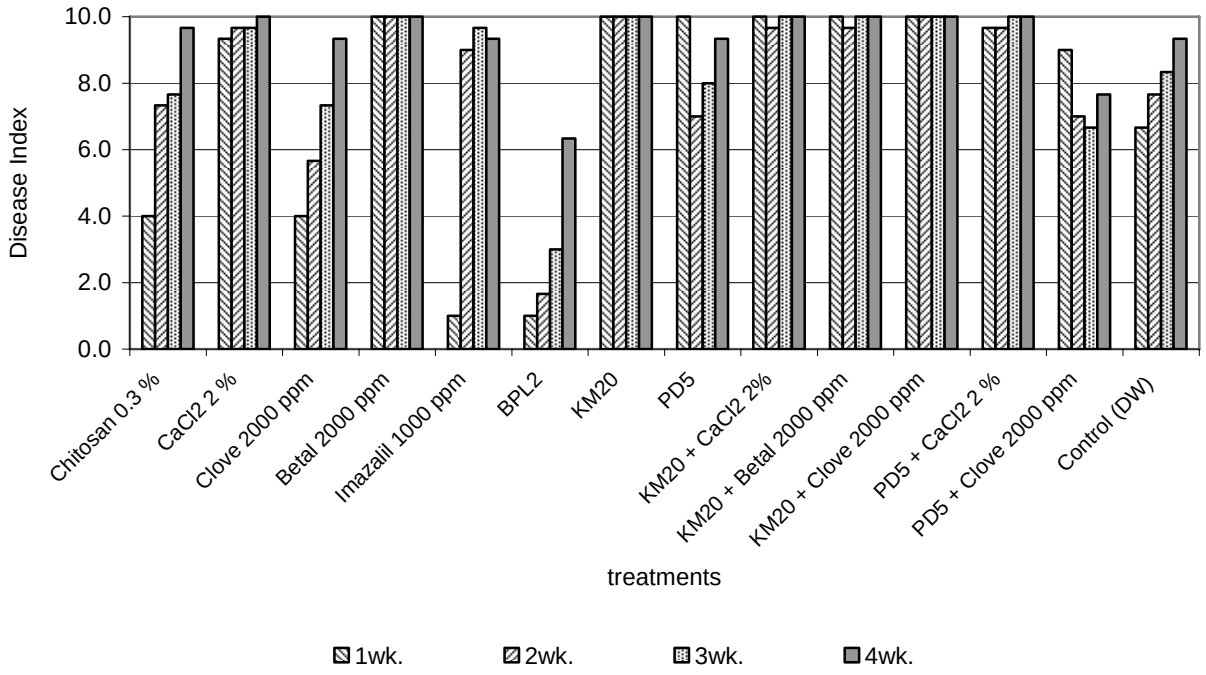

Figure 18 Disease index of ginger rhizome treated with compounds (Chitosan, $CaCl_2$) clove and betal vine leave extracts and antagonistic bacteria 'BPL2' and antagonistic fungi 'KM20' 'PD5' compared to fungicide Imazalil at 1-4 weeks under storage condition (13 °C, 65 % RH).

Note : Disease index (DI) of ginger rhizome rot, mean :

1 = no infection	6 = 50 % of rhizome was infected
2 = 10 % of rhizome was infected	7 = 60 % of rhizome was infected
3 = 20 % of rhizome was infected	8 = 70 % of rhizome was infected
4 = 30 % of rhizome was infected	9 = 80 % of rhizome was infected
5 = 40 % of rhizome was infected	10 = > 90 % of rhizome was infected

Table 13 Disease index of ginger rhizome treated with Chitosan, clove and antagonistic bacteria 'BPL2' compared to fungicide Imazalil at 1-4 weeks under storage condition (13 °C, 65 % RH).

Treatment	Disease Index (DI)			
	1st wk.	2nd wk.	3rd wk.	4th wk.
Chitosan 0.3 %	0.0[1]	7.0	9.7	9.7
Clove 2,000 ppm	3.3	9.7	10.0	10.0
Imazalil 1,000 ppm	0.0	0.0	0.0	0.0
BPL2	0.3	3.0	3.7	6.0
Chitosan 0.3 % + Imazalil 1,000 ppm	0.0	0.0	0.0	0.0
Control (DW)	3.7	10.0	9.0	8.7

[1]Average from 3 replications

Note : Disease index (DI) of ginger rhizome rot, mean :

1 = no infection
2 = 10 % of rhizome was infected
3 = 20 % of rhizome was infected
4 = 30 % of rhizome was infected
5 = 40 % of rhizome was infected
6 = 50 % of rhizome was infected
7 = 60 % of rhizome was infected
8 = 70 % of rhizome was infected
9 = 80 % of rhizome was infected
10 = > 90 % of rhizome was infected

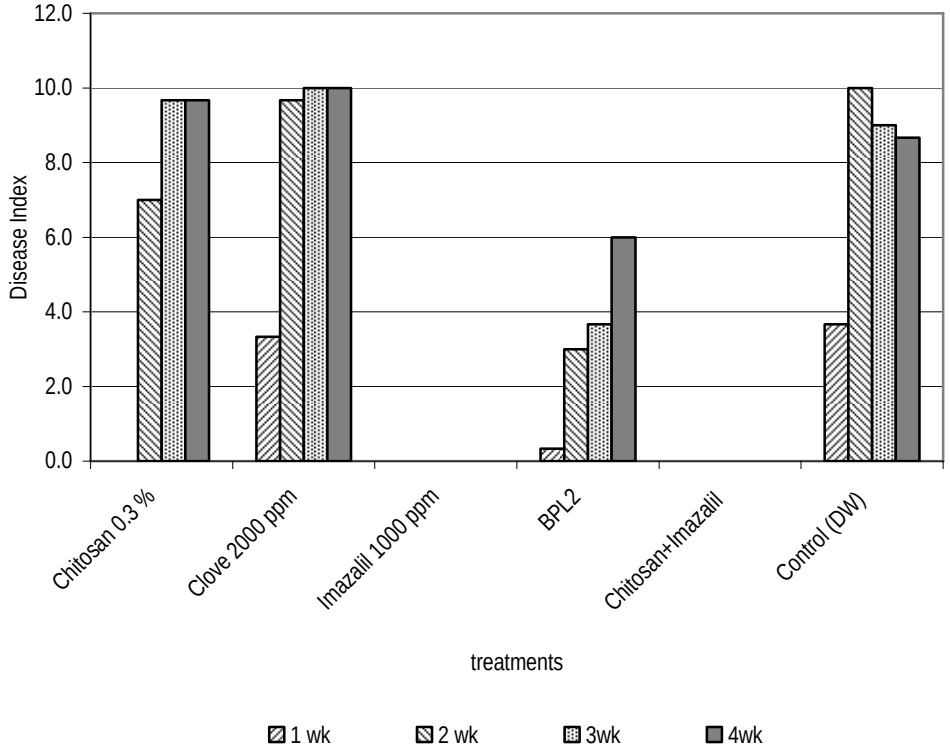

Figure 19 Disease index of ginger rhizome treated with Chitosan, clove and antagonistic bacteria 'BPL2' compared to fungicide Imazalil at 1-4 weeks under storage condition (13 °C, 65 % RH).

Note : Disease index (DI) of ginger rhizome rot, mean :

1 = no infection	6 = 50 % of rhizome was infected
2 = 10 % of rhizome was infected	7 = 60 % of rhizome was infected
3 = 20 % of rhizome was infected	8 = 70 % of rhizome was infected
4 = 30 % of rhizome was infected	9 = 80 % of rhizome was infected
5 = 40 % of rhizome was infected	10 = > 90 % of rhizome was infected

Table 14 Disease index and decaying percentage of ginger rhizome treated with clove extract and antagonistic bacteria 'BPL2' compared to fungicide Imazalil at 4 weeks after treatment under storage condition (13 °C, 65 % RH).

Treatment	Disease index		Decay (%)	
Imazalil 1,000 ppm	0.03[1]	c[2]	0.0	c
Clove 2,000 ppm	7.67	a	49.6	a
BPL2	4.33	b	8.4	b
Control (DW)	8.03	a	48.2	a

[1]Average from 3 replications.

[2]Values followed by a common letter are not significantly difference at the 5% level by DMRT.

4. Influencing factors of inducing wound healing on ginger for controlling of rhizome rot during storage.

4.1 Effect of healing conditions on rhizome rots control.

The results of *Fusarium oxysporum* infection on treated ginger rhizomes under high relative humidity condition were observed at 0, 5 and 8 DAI. (Figure 20). The ginger rhizomes treated by high relative humidity condition at every incubating temperature showed obviously resistance to *Fusarium oxysporum* infection at 8 DAI, otherwise, the ginger rhizomes treated under dry condition had completely infected.

The figures of cross section of wounded tissue of ginger rhizomes from every treatment of healing condition were conducted using microtome section and visually observed under stereo compound microscope. (Figure 21, 22) The figures 22 D, E and F, of those treatments of ginger rhizomes incubated under high relative humidity condition for 48 hrs at 15 ºC, 28±3 ºC (room temperature) and 32 ºC, showed the formation of layer at the edge of plant tissue while the others was disappeared.

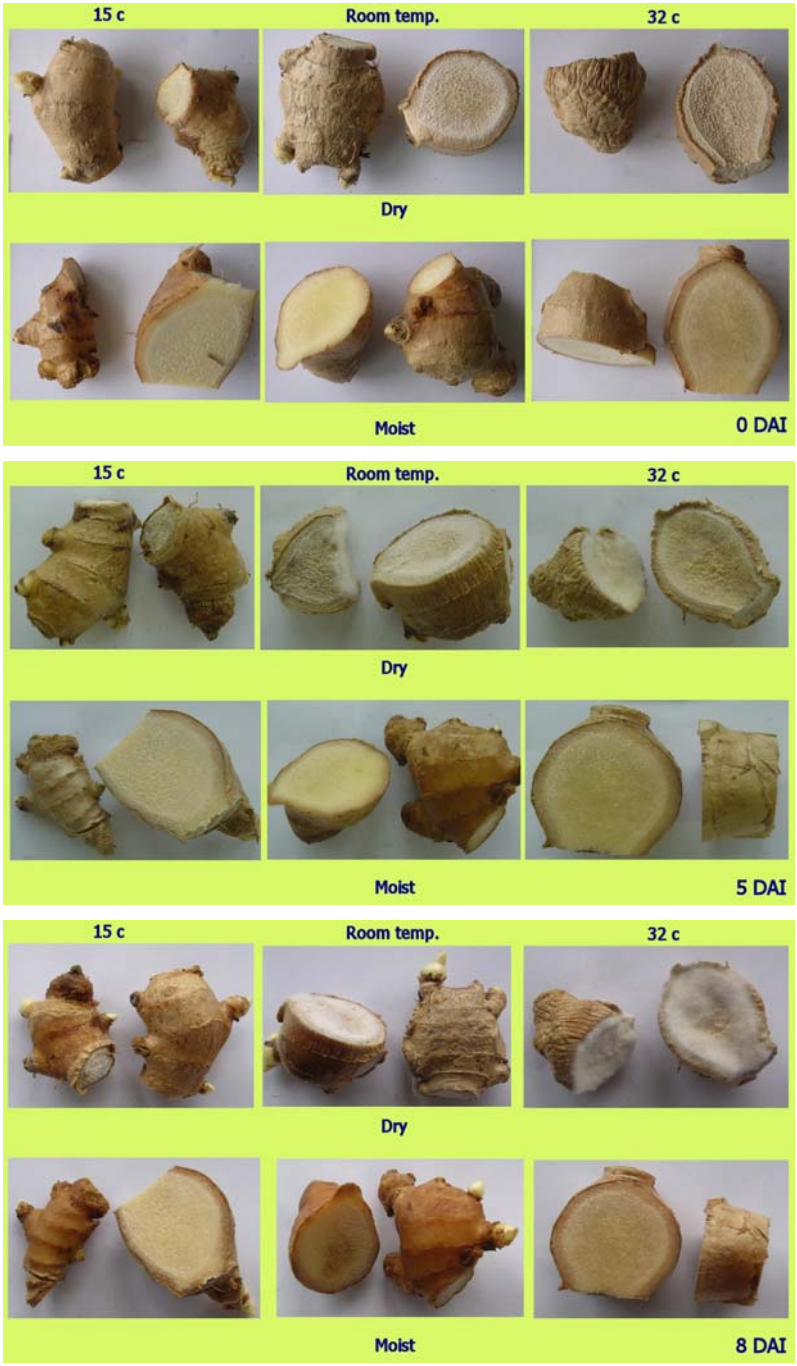

Figure 20 Infection of *Fusarium oxysporum* on ginger rhizome which combined treated between healing conditions, 3 levels of temperature (15 ºC, 28±3 ºC (Room Temp.) and 32 ºC), and high relative humidity condition (open air and moist chamber) for 48 hr. then incubated in high relative humidity chamber and the results were observed at 0, 5 and 8 DAI.

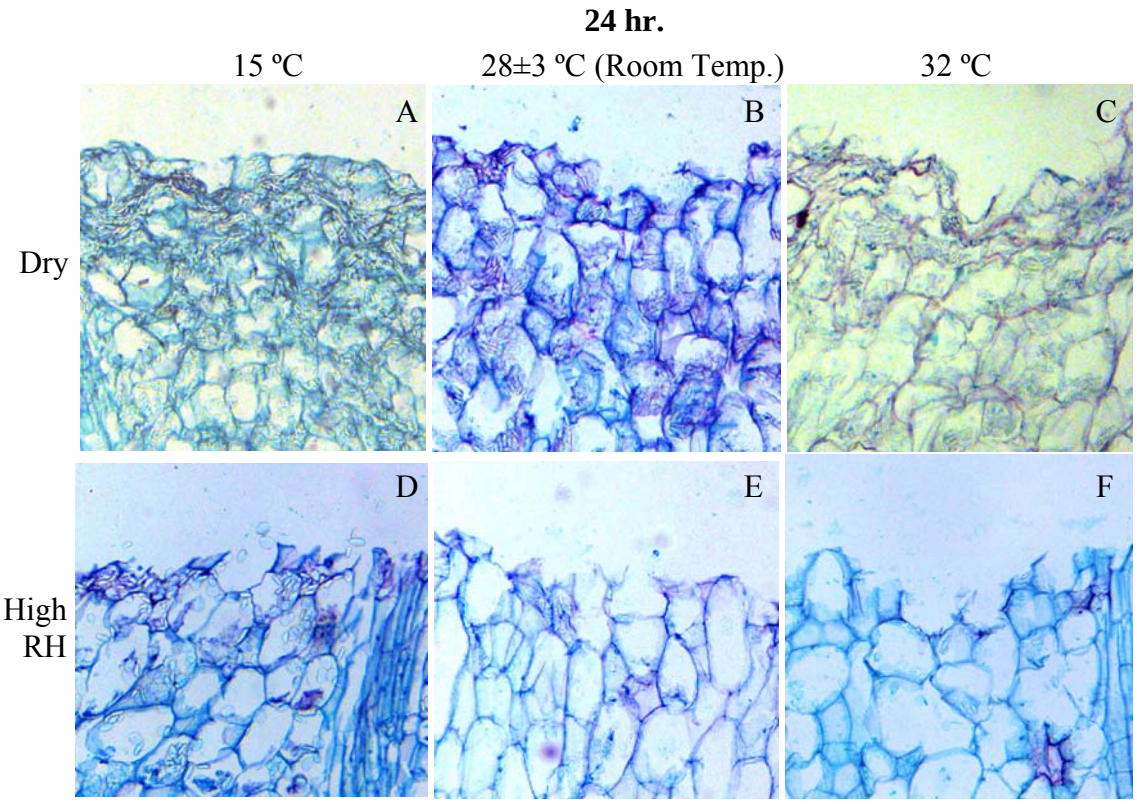

Figure 21 Cross section of ginger rhizomes tissue at 24 hr. after healing incubation which combined treated between healing conditions, 3 levels of temperature 15 ºC (A, D), 28±3 ºC (Room Temp.) (B, E) and 32 ºC (C, F), and, open air (A, B, C) and high relative humidity condition (D, E, F) under light microscope (x100).

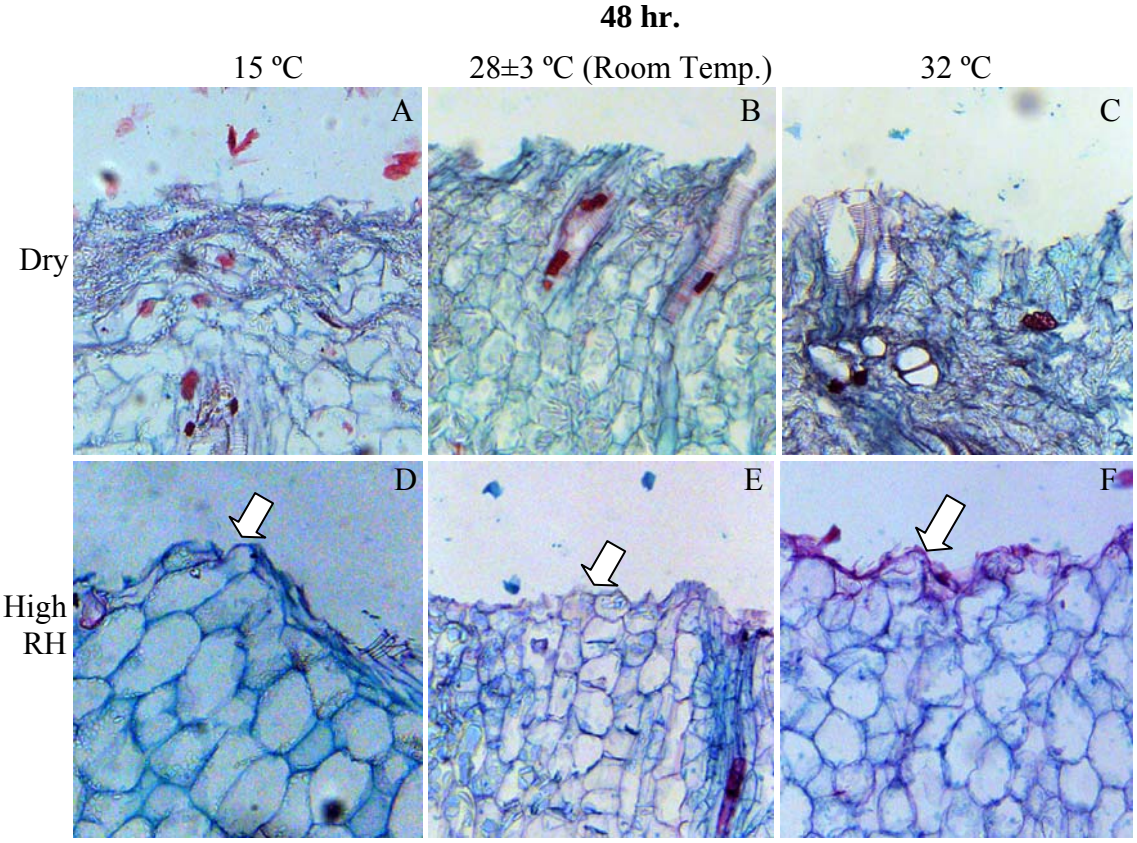

Figure 22 Light microscopy (x100) of cross section of ginger rhizomes tissue at 48 hr after healing incubation which combined treated between healing conditions, 3 levels of temperature 15 ºC (A, D), room temperature 28±3 ºC (B, E) and 32 ºC (C, F), and incubated under open air (A, B, C) and high relative humidity conditions (D, E, F).

4.2 Controlling of ginger rhizome rot during storage by using healing conditions.

The ginger rhizome was treated with various storage conditions compared to fungicide control treatment, 1,000 ppm Imazalil. The results showed that healing ginger rhizome (H^+) kept in plastic bag without holes gave the lowest disease index except 1,000 ppm Imzalil, disease index were scored 2.25 and 1.25 respectively (Table 15, Figure 23).

The percentage of weight loss of healing ginger rhizome (H^+) kept in plastic bag without holes at 42 DAT was low percentage of weight loss, 2.88 %, while those treatments without plastic bag wrapped found high percentage of weight loss (Figure 24).

Table 15 Disease index (DI) and percentage of weight loss of ginger rhizome treated with healing incubation (H^+, H^-) at different storage conditions, wrapped plastic bag with/without holes and plastic chamber, compared to 1,000ppm Imazalil fungicide (Imz^+, Imz^-) at 7-42 DAT (Day After Treatment) under storage conditions (13 °C, 65 % RH).

Treatment	Disease index at DAT [1]						weight loss
	7	14	21	28	35	42	(%)
Imz^+, H^- ($Check^{neg}$)	0.00b[1/]	0.13d	0.13f	0.50e	0.75e	1.25f	32.72a
Imz- H- ($Check^{pos}$)	0.25ab	3.75a	6.75a	7.50a	9.25a	8.00a	38.45a
H^-	0.50ab	1.00bc	1.50de	5.00b	6.25c	7.00ab	36.25a
H^- plastic bag w/o holes	0.37ab	0.75bcd	0.38f	0.75e	1.38e	2.25f	2.87d
H^- plastic bag with holes	0.12ab	1.13b	0.63ef	3.25c	4.25d	7.50a	9.12bcd
H^- plastic chamber	0.25ab	3.25a	5.00b	4.00c	7.75b	5.25cd	17.9b
H^+	0.25ab	0.88bc	1.50de	2.00d	5.75c	4.25de	35.3a
H^+plastic bag w/o holes	0.62a	0.75bcd	0.75ef	3.50c	3.25d	4.50cde	7.55cd
H^+plastic bag with holes	0.25ab	0.38cd	2.75c	3.25c	4.25d	5.75bc	11.35bcd
H^+plastic chamber	0.00b	0.88bc	1.88d	2.25d	6.00c	3.75e	14.6bc
% CV	48.26	33.06	26.48	19.76	15.25	18.25	28.90
F-test	ns[2]	***[3]	***	***	***	***	***
$LSD_{0.05}$	0.531	0.614	0.812	0.913	1.077	1.305	8.604
p- value	0.346	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001

[1]Values followed by a common letter are not significantly different at the 5% level by DMRT.

[2]ns = not significantly

[3]*** = highly significantly

Disease index (DI) : 0-9

0 = healthy

9= heavily infection (rotten)

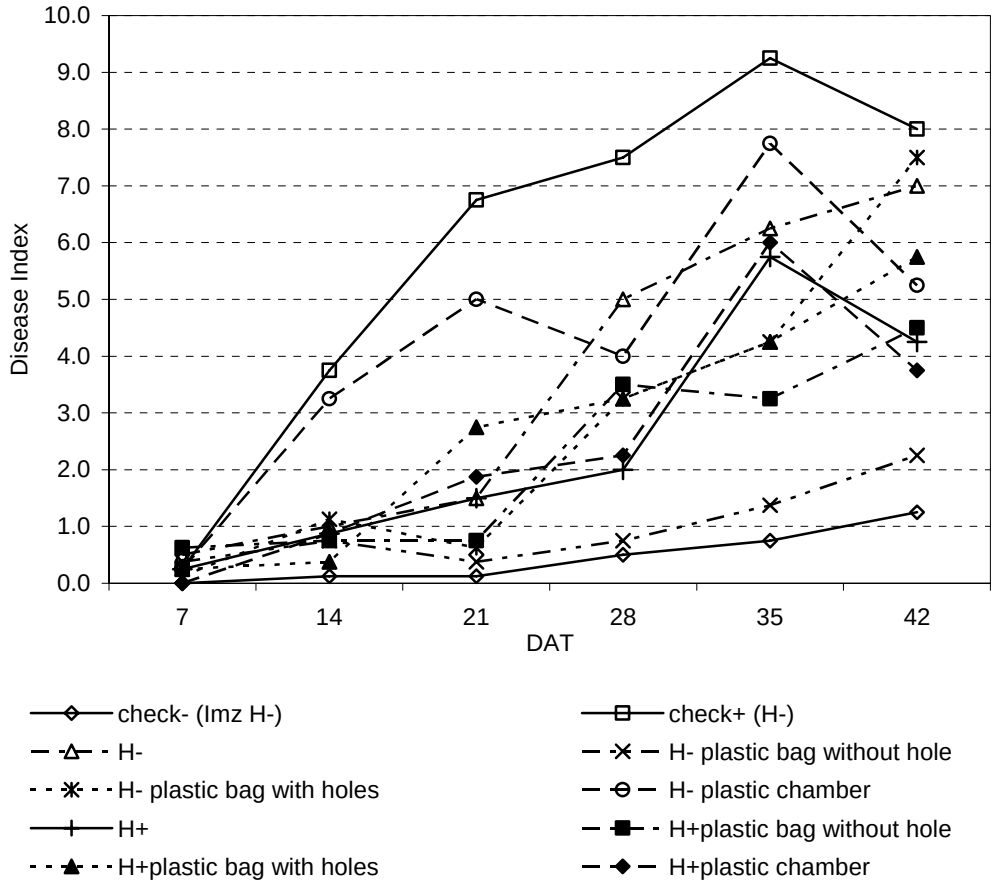

Figure 23 Disease index of ginger rhizome treated with healing incubation (H+, -) different storage conditions, wrapped plastic bag with/without holes and plastic chamber, compared to 1,000 ppm Imazalil fungicide (Imz +, -) at 7-42 DAT (Day after Treatment) under storage condition (13 °C, 65 % RH). Disease index; 0-9 (0 = healthy rhizome 9= completely decayed).

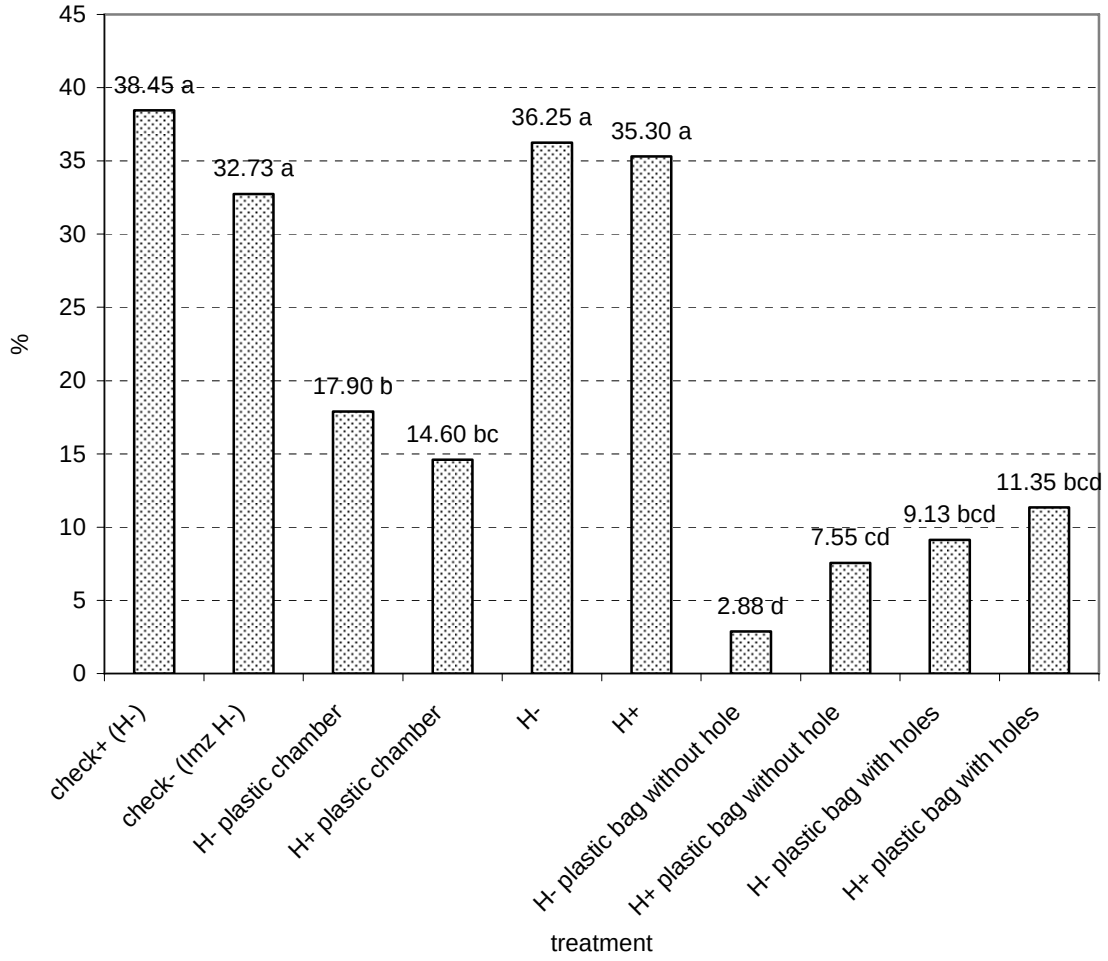

Figure 24 Percentage of weight loss of ginger rhizome treated with healing incubation (H+, -) different storage conditions, wrapped plastic bag with/without holes and plastic chamber, compared to 1,000 ppm Imazalil fungicide (Imz +, -) at 7-42 DAT (Day after Treatment) under storage condition (13 °C, 65 % RH). Disease index scored; 0-9 (0 = healthy rhizome 9= completely decayed).

(Values followed by a common letter are not significantly different at the 5% level by DMRT.)

Discussion

The isolation of pathogenic fungi of ginger rhizome rot during storage using tissue-transplanting technique was conducted. The causal fungi was cultured and identified as *Fusarium oxysporum*. This identified pathogen was also reported during exportation by Tanboon-Ek *et al.* (1978). Chantaraotan *et al.* (1986) reported that *Fusarium* sp. is pathogenic fungi of ginger rhizome rot in the fields, but it is not vilurence as pathogenic bacteria. Whereas *Fusarium oxysporum* was also isolated from ginger samples those collected from markets and fields (Rath and Misha, 1993). The information revealed that the pathogen, *Fusarium oxysporum* are generally distributed in the ginger fields but it does not the major infectious fungi. The inoculums of *F. oxysporum* was contaminated on rhizome through post-harvest handling processes, whenever the storage conditions, low temperature and high relatives humidity, are optimized to fungal growth, they will grown up and established on the rhizome, especially wounded tissue. Doroo (1989) reported that the consistence information of the aggressiveness of infection of ginger rhizome rot during storage is positively correlated to the infection of *F. oxysporum* f.sp. *zingiberi* in the field.

Three hundred and twenty-one isolates of microorganism were introduced to screening test for potential antagonistic with pathogen, *F. oxysporum*. Forty-seven fungi isolates and 17 bacterial isolates were showed the antagonistic potential in different interaction among, parasitism competitive and antibiosis. Thereafter, antagonistic microorganisms those had properties of competitive potential and survival on rhizomes, positively responded to compounds and tolerated to plant extracts were selected to use as a biological control agent on ginger rhizomes under post-harvest conditions.

Two antagonistic *Trichoderma* ‘KM20’ and ‘PD5’ were selected for the next experiment in order to their ability of growth and compete to the pathogen for nutrition and space competitiveness, highly tolerance to 50,000 ppm of clove extract and moderate tolerance to 50,000 ppm of betal vine leave extract. Whereas isolate ‘MJ4’ was highly tolerance to 100,000 ppm of clove and betal vine leave extract and

highly responsible to compounds $CaCl_2$ and D-fructose, nevertheless, the vegetative growth was slower than ‘KM20’ and ‘PD5’. Antagonistic bacteria, isolates ‘BKM10, BKM8 and BPL1’ tolerated to both plant extract but only ‘BPL2’ was responded to the compounds D-fructose and $CaCl_2$. Ghout *et al.* (2000b) found that combined using of *Candida saitoana* and 0.2 % of 2-Deoxy-D-Glucose before inoculation gave the best efficiency to control decaying of apples lemons and oranges during storage compared to merely using. However, using the combination after 24 h inoculation gave inhibition on blue mold of apples, green mold of oranges and lemons equal to using of imazalil. 2-Deoxy-D-Glucose which added into the culture of *C. saitoana* had affected on growth rate of yeast but not on wound tissue neither the presents or absences of 2-Deoxy-D-Glucose.

Two isolates of antagonistic *Trichoderma* ‘KM20’ and ‘PD5’ were selected to test for growth on ginger rhizome. The results showed the selected isolates of fungi able to grow and survive on ginger rhizome, especially on wounded tissue. However, the firmness of wounded tissue was decreased by infection of antagonistic *Trichoderma* ‘KM20’.

In vitro test for antibiosis of antagonistic microorganism on growth of pathogen revealed that antagonist, *Trichoderma* ‘KM20’ and ‘PD5’ antagonistic bacteria ‘BPL2’ did not produce antibiotic to inhibit vegetative growth of *F. oxysporum*. Therefore, the interaction of antagonist was not the antibiosis.

The efficacy of 95 % ethanol crude extracts of thirty-one species of medicinal plant on vegetative growth inhibition of *F. oxysporum* were conducted by using filter paper disc method, only betal vine leave (*Piper betal* L.) and clove flower (*Syzygium aromaticum* (Linn.) Merr & Perry.) crude extracts concentration at 100,000 ppm were showed inhibitory effect on *F. oxysporum* by clear zone diameter of 2.45 and 2.05 cm, respectively. The clear zone diameters of two plant extracts are 78.27 and 65.49 % respectively, compared to the fungicide Immazalil at concentration 1000 ppm. Dilokkunanant *et al.* (2000) reported as the same result of inhibitory effect of 95 % ethanol extract of betal vine leave on *Fusarium* spp. at concentration 100,000 ppm.

The investigation of the polarity properties of active compound in plant extracts using the solvent partition. The result showed that aqueous1fraction of betal vine leave extract provided the largest clear zone at concentration 100,000 ppm, 36.21 % compared to Imazalil 1,000 ppm, whereas petroleum ether fraction of clove extract provided the clear zone 32.67 % at concentration 100,000 ppm. The solvent partition revealed that active compound in clove was low polarity compound while the betal vine leave was moderate polarity compound.

The population change of antagonistic *Trichoderma* 'KM20' and 'PD5' were increased rapidly as same as the pathogen, especially on wounded tissue. Whereas the population change on skin were slightly increased at initial of incubation period and consistent in change until the end of incubation period. The results revealed that competition ability of antagonist is the important factor to control the infection of pathogen especially, on the wounded tissue of ginger rhizome.

The efficacy of betal vine leave and clove extracts on decreasing of *F. oxysporum* population on ginger rhizome at the initial of incubation period were observed. Thereafter it was increased suggested that the efficacy of those extracts exhibited only the inhibition growth of *F. oxysporum* but not eradication.

The non-toxic compounds, D-fructose $CaCl_2$ and Chitosan, were introduced to test for enhancing ability of antagonist on controlling of *F. oxysporum* by using poisoned food technique. Chitosan showed inhibitory effect on vegetative growth of *F. oxysporum* in all tested concentration, 0-0.6 %, while D-fructose and $CaCl_2$ promoted vegetative growth at low concentration, 0.5-2.0 %. At 0 % of Chitosan the percentage of inhibitory effect was 22.0 %, it might be due to the acidity in culture media, because Chitosan was dissolved in 1 % acetic acid before added into culture media. Benhamou and Theriault (1992) reported that the effect of chitosan on the induction of host cell reactions was observed at concentration from 0.5 to 2 mg/ml with an optimal effect at 2 mg/ml. To enhance protection of tomato roots to fungal

attack upon application of chitosan to leaves suggests that chitosan-induced resistance is systemic.

Janisiewiez *et al.* (1998) reported that fructose was not inhibit the vegetative growth of *Botrytis cinerea* the pathogen of post-harvest decay of apple fruits, but it promoted the efficiency of yeast '*Metschnikowia pulcherrima*' to inhibits apple decay. While, Arras *et al.* (1997) reported that 4 % of $CaCl_2$ gave 49 % growth inhibition of *Pennicilium italicum* on orange fruits. Moreover, Benhamou (1992) reported that Chitosan was able to inhibit growth of *F. oxysporum* f. sp. *radicis-lycopersici* at concentration 3-6 mg./ml., the fungal mycelium was swollen and the activities of β-1,3-glucanase and chitosanase was stimulated.

The three of compounds, plant extracts, antagonistic fungi and bacteria were introduced to *in vivo* test on controlling ginger rhizomes rot under storage condition. In this study, there were 3 experiments to screening the unaccepted treatments. The first experiment was conducted with 14 treatments included positive and negative control treatments. The results revealed that those treatments consisting of antagonistic *Trichoderma* 'KM20' and 'PD5' had greenish fungal mycelium grown on wounded tissue were not selected to use in the following experiment. In the second experiment, acceptable treatments Chitosan, Clove extract and antagonistic bacteria 'BPL2' were selected for the further studied. The results of this experiment revealed that antagonistic bacteria 'BPL2' provided the lowest disease index therefore this antagonistic bacteria was selected to test in semi-commercial experiment. The semi-commercial experiment was conducted with the treatment of antagonistic bacteria 'BPL2' and results showed that 'BPL2' gave the lowest disease index of 4.33 and percentage with 8.4 %, of rhizome decay. While Clove 2,000 ppm and control treatment gave disease index of 7.67 and 8.03 and percentage of rhizome decay were 49.6 and 48.2 %, respectively.

In the study of wound healing of ginger rhizome, revealed that ginger rhizome incubated for 48 hrs under high relative humidity condition showed the resistance to *F. oxysporum* infection, whereas, rhizome was susceptible of infection at dry

condition. The cross section of rhizome tissue showed that the structure layer alike was created at the wounded tissue. Lulai (1998) reported that there were small changes in lipoxygenase (LOX) activity within 24 h after wounding in some potato cultivars. Two to three days after wounding the changes in LOX activity accelerated and generally reached a max during the 5th to 7th day. During healing, the tissue increased in total soluble protein which paralleled to the LOX activity. The LOX wound respond to the impact and scuffing injury on the surface of whole uncut tubers then transmitted into the interior of the tuber. During the wound healing period, LOX activity from the vascular ring inward was lower than that found from the vascular ring outward to the tuber surface; however, the activity increased in these 2 tissue areas were parallel. The changes in LOX activity in response to tuber injury implied that the LOX metabolic pathway(s) could have a role in wound healing and/or plant defense. Thomson *et al.* (1995) reported that both lignin and the prosuberin lamella were first detected in wound reaction cells of the cortex at 4 h in cut, core, disc, and slice wounds and at 8 h in bruise wounds. Healing was completed with formation of a continuous wound cork cambium beneath the wound site. With lignifications and suberization, wounded potato tubers apparently develop specialized barriers against potential pathogens and water loss. The overall healing process proceeds more slowly in bruise wounds than in cut wounds. Moreover, the capacity for wound healing decreases with increasing age of the tuber, or time during storage.

While, Hawkins and Boudet (1996) said those 3 days after wounding, a conspicuous "barrier" formed by the hypertrophy dedifferentiation of parenchyma cells in the bark wound zone. This barrier (developing wound periderm) extends from the epidermis (including epidermal cells) through the cortex and phloem to the vascular cambium where it joins up with the xylem tissue exposed at the initial wound site. It appears to be one, or at the most two, cell(s) thick and separates underlying sensitive tissues from the environment. Histo-chemistry and subsequent fluorescence microscopy suggest that the cell walls making up the barrier are both lignified and suberized.

The studies by treatments of different healing and storage condition were conducted with various storage conditions compared to fungicide control treatment, 1,000 ppm Imazalil. The results showed that healing ginger rhizome before kept in plastic bag without holes provided the lowest disease index (2.25) except fungicide Imazalil (1.25) and also provided the lowest percentage of weight loss (2.88 %). However, the condition of highly relative humidity would rather effect to card boxes that containing the rhizome, consequently, it was soaked with moisture and some rhizomes were emerging during of experiment. Lulai and Suttle (2004) reported that in potato tuber, ethylene is not required for wound-induced suberization of the closing layer (suberization of existing cells at the wound surface) during the first 2-4 days of wound-healing or subsequent suberization of phellem cells (between 4 and 9 days) created by the wound-induced formation of the phellogen.

In the future, the conditions of healing and storage of ginger rhizome to avoid the discourage defects and safe to the consumers need to be studied. The mechanisms of wound healing for rhizome rot resistance must be clarified.

Conclusion

The causal microorganism of ginger rhizome rot during storage was investigated and identified as *Fusarium oxysporum*. Forty-seven isolates of fungi and 17 isolates of bacteria from 321 isolates of microorganism showed antagonistic ability on controlling of the causal fungi.

To evaluate the efficacy of medicinal plant crude extracts, 31 plant extracts were introduced to test with causal fungi, only betal vine leave (*Piper betal* L.) and clove (*Syzygium aromaticum* (Linn.) Merr & Perry.) crude extracts were showed inhibitory effect on *Fusarium oxysporum*. The percentages of growth inhibition were 78.27 and 65.49 % at 100,000 ppm of extracts, compared to 1,000 ppm of Imazalil, respectively. The efficacy evaluation of partially purified of plant extracts was conducted, the most active compound of betal vine leave extract are in aqueous1 and the clove extract are in petroleum ether fraction. The solvent partition with petroleum ether and ethyl acetate revealed that active compound in clove was low polarity compound while the betal vine leave was moderate polarity compound.

To enhance the efficacy of antagonistic microorganisms, three compounds, namely D-fructose $CaCl_2$ and Chitosan, were tested by poisoned food technique on vegetative growth of *F. oxysporum.* The results showed that Chitosan 0.6 % provided the highest growth inhibition with the percentage of 75.33 %, whereas $CaCl_2$ 4 % provided 66.67 %. The concentrations of $CaCl_2$ lower than 1 % and all concentration of D-fructose (0.5 to 4 %) could not inhibited vegetative growth of the pathogen.

Antagonistic *Trichoderma* 'KM20' and 'PD5' and antagonistic bacteria 'BPL1' were tolerated to betal vine leave and clove extracts and responsible to $CaCl_2$, hence, those were selected to studies on controlling of ginger rhizome during storage. The antagonistic bacteria 'BPL1' provided the lowest disease index except fungicide Imazalil. The semi-commercial experiment also suggested that antagonistic bacteria 'BPL1' provided the lowest disease index except fungicide Imazalil by 8.4 % of rhizome decay.

The ginger rhizome incubated for 48 hrs under high relative humidity condition showed the resistance to *F. oxysporum* infection, whereas, in dry condition the rhizome was susceptible to infection. The study of different healing and storage condition, revealed that healing before wrapping by plastic bag without holes provided the lowest disease index (2.25) and weight loss (2.88 %) except 1,000 ppm of fungicide Imazalil (1.25).

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APPENDIX

1 *Trichoderma* selective medium I (Dhingra, 1995)

Glucose	3.0	g
NH_4NO_3	1	g
K_2HPO_4	0.9	g
KCl	0.15	g
$MgSO_4.7H_2O$	0.2	g
Agar	15	g
Water	1	l

After autoclaved add chloramphenicol 250 mg fenaminosulf 300 mg quintozene 200 mg captan 20 mg and rose bengal 150 mg

2 *Fusarium* selective medium V (Nash & Snyder medium) (Dhingra, 1995)

Peptone	15	g
$MgSO_4.7H_2O$	0.5	g
KH_2PO_4	1	g
Agar	20	g
Water	1	l

After autoclaved add Quintozene1 g and Streptomycin 300 mg

3. Potato Dextrose Agar (PDA)

Potato	200	g
Dextrose	20	g
Agar	12	g
Distilled water	1,000	ml

4. Nutrient Agar (Difco 0001)

Beef extract	3.0	g
Bacto poptone	5.0	g
Agar	15.0	g
Distilled water	1,000	ml

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SCOLARSHIP