CHAPTER I

INTRODUCTION

Fungi are widely distributed in nature and are frequent pathogens of various species in the plant kingdom, representing one of the main causes responsible for crop losses. Therefore, many effective fungicides have been developed, commercialized and applied to the field, but diseases induced by fungi localized in the tissues, curative treatments are difficult to carry out. A strategy may consist in identifying natural molecules with antifungal properties which can be transported over long distances in the plant towards the fungal target.

Considering their known chemical and biological properties, phenolics might fulfill the requirements of such a strategy. Phenolics such as many benzoic acid derivatives accumulate in plants during the phase of infection. These compounds result from the activation of the key enzyme, phenylalanine ammonia lyase, leading from phenylalanine to the formation of various derivatives (Amborabe, 2002).

1.1 Plant diseases caused by fungi

Plant diseases caused by fungi, bacteria, viruses and other microorganisms, are a leading cause of agricultural crop loss. One of the most important plant diseases in the world is fungi. Fungi are highly variable group of organisms which do not contain chlorophyll and conductive tissues. The most of fungi have filamentous vegetative structures (mycelium) and they usually reproduce by spores. More than 8,000 species of fungi cause diseases in plants (Agrios, 1997). Many plant diseases are recognized by characteristic symptoms on host plants. More specific identification of disease may require observations of characteristics of the causative pathogen. Fungi are identified by their spores and fructifications (fruiting bodies), also called spore-bearing structures.

In this research, four pathogenic fungi *Alternaria porri*, *Fusarium oxysporum*, *Pestalotiopsis* sp. and *Phytophthora parasitica*, causing disease in many Thai crops, were selected as model organisms.

1.1.1 Plant diseases caused by *Alternaria porri*

Purple blotch of onion (*Allium cepa* L. var. *cepa*) is caused by *A. porri*. This fungus is also a pathogen of garlic (*Allium sativum* L.), shallot (*Allium cepa* L. var. *ascalonicum*) and leek (*Allium porrum* L.). *A. porri* spores develop in high humidity and can spread to onion foliage and bulbs in the field or storage shed by wind, water splashing, implements and insects or workers.

Alternaria spores germinate on onion leaves and produce a small, water-soaked spot that turns brown. The elliptical lesion enlarges, becomes zonate (target spot) and purplish. The margin may be reddish to purple and surrounded by a yellow zone. During moist weather, the surface of the lesion may be covered by brown to black masses of fungal spores.

Lesions may merge or become so numerous that they kill the leaf. Leaves become yellow then brown, and wilt downward two to four weeks after initial infection. Lesions may form on seed stalks and floral parts of seed onions and affect seed development. Spores may be blown or washed down to the neck region and infect the outer scales of bulbs through wounds or the neck tissue. A yellow to wine-red, semi-watery decay may occur. Diseased tissue turns brown to black and dries out in the field or, more commonly, in storage.

Downy mildew symptoms appear on older leaves as elongated patches that vary in size and are slightly paler than the rest of the foliage. With moisture, these areas become covered with a violet-gray mycelium, which contains spores that may be spread to surrounding healthy tissue. The oval lesions may be violet to purple and may be confused with the initial elongated lesions of purple blotch. Affected leaves gradually become pale green and later yellow. Diseased parts, such as leaf tips, fold over and collapse. Systemically infected bulbs become soft, shriveled and watery.

A. porri has dark-colored mycelium and in older tissue it produced short, simple, vertical conidiophores that produce single or branched chains of conidia. The conidia are large, dark, long or pear shaped and multicellular with both horizontal and longitudinal cross walls. The conidia are detached easily and are carried by air currents. *Alternaria* spores are one of the most common fungal caused of allergies (Chavarin, 2002).

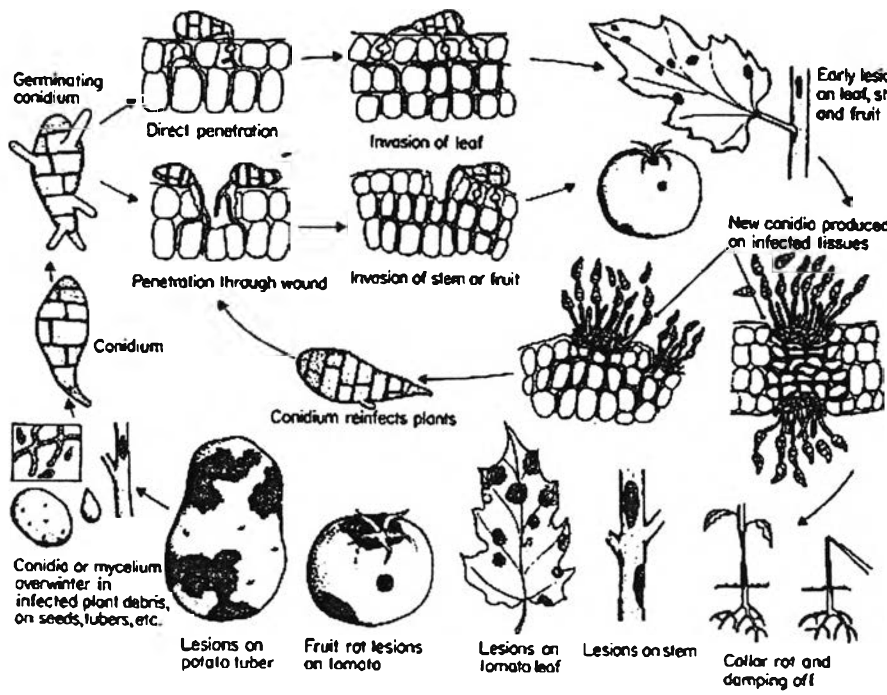

Fig 1.1 Disease cycle of *Alternaria* sp. (Agrios, 1997)

The development of disease (Fig 1.1) caused by *Alternaria* initiates from mycelium and spores infest in plant debris, on seeds and tubers. The mycelium germination is direct penetration or penetration through wound and leaf or stem are attacked and the symptom of disease will be present as collar rot damping off, stem rot, leaf rot and fruit rot.

1.1.2 Plant diseases caused by *Fusarium oxysporum*

F. oxysporum species complex gathers diverse soil-borne (survival of a pathogen from year to year in soil) fungi including plant pathogens causing vascular wilt on more than 100 cultivated plants (Khang, 2005), very injurious and frightening plant disease appearing as more of less rapid wilting, browning and dying of leaves, shoots followed by final death of whole plant. Wilts occur from the presence of the pathogen in xylem vascular tissues. Some vessels may be clogged with mycelium, spores or polysaccharide produced by the fungus. Clogging is further increase by accumulation of breakdown product of plant cells digested by fungus enzyme. The oxidation and translocation of breakdown products seem to also be responsible for the brown discoloration of affected vascular tissue (Agrios, 1997). The pathogen usually continues to spread internally through the xylem vessels until the entire plants are killed. The fungus infects the plants through the roots *via* direct penetration or *via* wounds, after which the xylem vascular tissue of the plant is colonized. The entire plant or the parts above the point of vascular invasion of the pathogen may die within a period of weeks after infection (Dardari et al., 2004). *F. oxysporum* can attack the plant at any stage of crop development. Symptoms are more noticeable if the crop is exposed to unfavorable conditions, such as drought and heat (especially with wilt diseases) or waterlogging.

The mycelium of *F. oxysporum* is colorless but become cream-colored or pale yellow with age. At optimum conditions it produced a pale pink or purplish colony. *F. oxysporum* produced three kinds of asexual spores, microconidia, which are one or two cell and usually produced under all conditions. Macroconidia are three to five cells and curve shapes. Chlamydospores are one or two cells, thick walled, round spores and produced terminally on old mycelium.

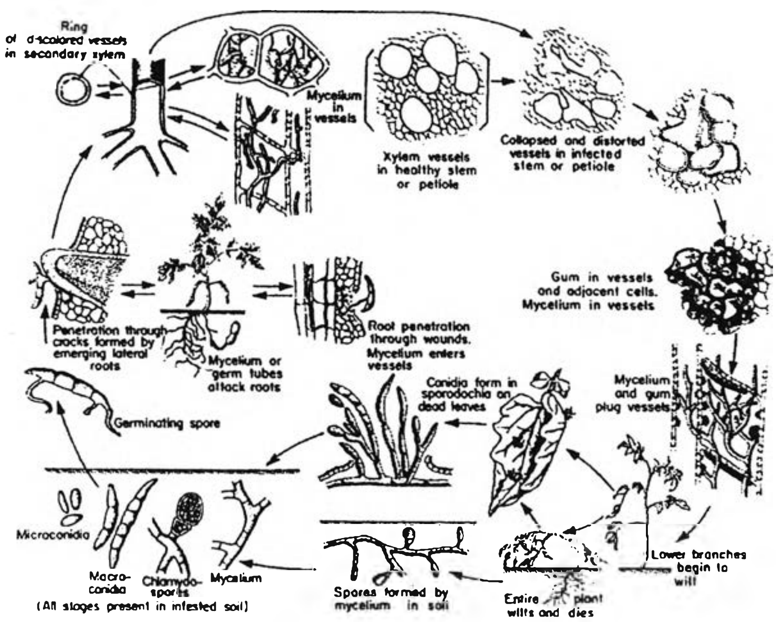

Fig 1.2 Disease cycle of *F. oxysporum* (Agrios, 1997)

F. oxysporum is a pathogen that causes tulip bulb rot and *F. oxysporum* is also reported to infect mustard (*Brassica campestris* L.), rice (*Oryza sativa* L.), spinach (*Spinacia oleracea* L.), and tomato (*Lycopersicum esculentum* Mill.) (Kuwaki, 2004). It has been reported that various *F. oxysporum* pathotypes can survive successfully either in the soil or above ground by means of thick-walled chlamydospores that are either free or embedded in infected plant debris (Vakalounakis, 2004).

The development of vascular disease shows in Fig 1.2. The pathogen infests in soil are mycelium and all spores form but the most commonly are chlamydospores. They spread by water or contaminated farm equipment. When plants grow in contaminated soil, the germ tube of spore or the mycelium sink in the root. The mycelium through the root cortex and enter the vessels. The ring of discolored vessels in secondary xylem will be present. After enlargement mycelium the cells were collapsed, vessele were distorted and plants will die.

1.1.3 Plant diseases caused by *Pestalotiopsis* sp.

Pestalotiopsis sp., is the main disease found to affect mangosteen (*Garcinia mangostana* L.) in the Thailand. The disease is thought to be predisposed by sunburn and often found after stormy conditions when limbs get damaged. *Pestalotiopsis* sp. is also a fungal disease that is considered usually a minor disease. It attacks foliage that has been injured or weakened by unfavorable weather or growing conditions. Usually, the killed foliage is near the base of the plant and where foliage is relatively dense. The disease usually starts at the tip of the foliage and progresses towards the leaf base. The color of the foliage goes from green to yellowish, then to a dark brown that can look almost black. The disease may kill the smaller twigs where the infected needles died. Pestalotiopsis sp. have usually been isolated as leaf and stem pathogens of economically important tropical plants such as the plams, pines, loquats, guavas, mangoes and a large number of ornamental plants (Li *et al.*, 2001)

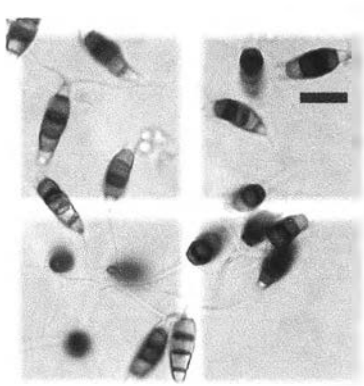

Fig 1.3 Spores of *Pestalotiopsis* sp.; bar = 10 µm

1.1.4 Plant diseases caused by *Phytophthora parasitica*

Phytophthora cause disease in many plants. Most species cause root rot, damping off of seedling, rot of lower stems, tubers, or may be cause rot of buds or fruits and foliar blights. The losses caused by *Phytophthora* are great, especially on trees and shrubs (Chavarin, 2002). Root rot of tomato (*Lycopersicon esculentum* Mill.) is caused by *P. parasitica.* The most distinctive symptoms of Phytophthora root rot are the brown lesions on roots of all sizes. The xylem of the roots above the lesions often turns yellowish or brown in color. In severe cases, nearly all roots may be girdled or rotted off. Aboveground, infected plants are slow growing and may wilt or die in hot weather. When fruit in contact with the ground are infected, the disease is called buckeye rot. Symptoms include tan or brown spots with concentric rings.

Phytophthora root rot suffusion where the soil becomes too wet, high soil moisture and atmosphere humidity, poorly drained area and temperature remain fairly low. Young seedling may be killed within a few days. While in older plant the killing of roots may be rapid or slow, depending upon the amount of fungus present in the soil and the prevalent environmental condition.

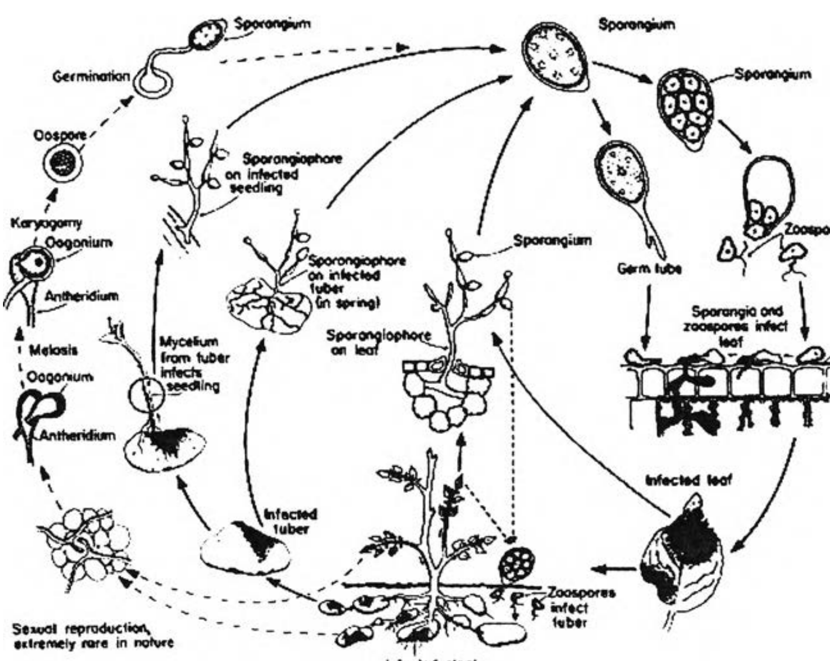

Fig 1.4 Disease cycle of *Phytophthora* sp. (Agrios, 1997)

The development of the disease is presented in Fig 1.4. The pathogen overwinters as oospore, chlamydospores. or mycelium in infest roots or in the soil. In the spring spore germination by means of zoospores, while the mycelium grows further and produces zoosporangia which release zoospores. The zoospores swim around and infect root of susceptible hosts. Furthermore the mycelium spreads up the stem in the cortical region causing discoloration and collapse of the cells. When the mycelium reaches the aerial part of plants, it produces sporangiophores, which emerge through the stomata of the leave and extend into the air. The sporangia produced on the sporangiophores become dispersed by rain. When the sporangia land on the wet soil they incite to start next cycle disease.

1.2 Literature search on the anmicrobial activity of benzoic acid derivatives

Following a pathogen attack, plants react by synthesizing a number of chemical defences in which the phenylpropanoids occupy a central place. It has been shown in a wide variety of host/pathogen relationships that the phenolic compounds may play a role in the resistance mechanisms of healthy plants. Their accumulation in infected tissues may also be implicated in the general mechanism of resistance against pathogens. Phenolic compounds such as many benzoic acid derivatives accumulate in plants during the phase of infection. Literature review showed that four benzoic acid derivatives, such as genuine acid, taboganic acid, methyl ester of 2,2-dimethyl-6-carboxychroman-4-one and methyl ester of 2,2-dimethyl-3-hydroxy-6-carboxychroman have been isolated from the dichloromethane extract of the leaves of *Piper dilatatum* (Piperaceae) displaying antifungal activity against *Cladosporium cucumerinum* in direct bioautography on TLC plates (Terreaux *et al*., 1998).

Among benzoic acid derivatives, salicylic acid acts directly on the fungal development in *Eutypa lata*, in a concentration-dependent manner, the threshold value being 0.1 mM. A fungicidal effect was obtained at 2 mM or higher concentrations and following this treatment, fungal filaments appeared empty. This action appears to be specific to the molecular arrangement, since a simple displacement of the hydroxyl group on the aromatic ring reduced the antifungal activity (Amborabe *et al*., 2002)

The 22 essential oil phenols, phenol ethers, and aromatic aldehydes have been tested for their antifungal activity against the wood-decaying fungi *Trametes versicolor* and *Coniophora puteana*. Thymol, carvacrol, *trans*-anethole, methyl chavicol, and cuminaldehyde as the main constituents of thyme, oregano, anise, basil, and cumin oils, were found to be the most effective compounds in inhibiting the growth of both wood-decaying fungi. The results also indicated that, a potential relationship between the chemical structure and the experimentally determined antifungal activity of the tested essential oil compounds against the two wood-decaying fungi. Compounds with more than one oxygen-containing group (–OH,–OCH_3, –CHO, –CO) in the aromatic ring exhibited less inhibitory activity against the tested fungi. Moreover, the position of the functional group in the aromatic ring was also shown to play an important role in the antifungal activity of the tested compounds. Functional groups in the second position of the aromatic ring decreased the antifungal activity (Voda *et al.*, 2003).

2,5-Dimethoxybenzoic acid was tested as an antifungal compound to control the postharvest decay pathogens of strawberry fruits. The compound completely inhibited *in vitro* spore germination and mycelial growth of *Botrytis cinerea* and *Rhizopus stolonifer* at a concentration of 5 mM (Lattanzio *et al.*, 1996)

Octyl gallate (3,4,5-trihydroxybenzoate) was found to possess antifungal activity against *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii.* The primary fungicidal activity of octyl gallate comes from its ability to act as a nonionic surfactant. The length of the alkyl chain is not a major contributor but plays an important role in eliciting the activity (Kubo *et al.*, 2001).

Antifungal activities of propyl (C_3), octyl (C_8) and dodecyl (C_{12}) gallates were tested against *Saccharomyces cerevisiae* and *Zygosaccharomyces bailii.* Octyl gallate was found to be the best active compound. *S. cerevisiae* produces acidification of the external medium during growth on glucose. The external acidification is closely associated with the metabolism of the sugar and also depends on the buffering capacity of the growth medium. The H^+-ATPase is important not only in the regulation of internal pH but also in the energy-dependent uptake of various metabolites. This glucose-induced medium acidification process was only inhibited by octyl gallate, indicating direct or indirect inhibition of plasma membrane H^+-ATPase (Fujita and Kubo, 2002).

Propyl gallate is widely used as an antioxidant in food and pharmaceutical products. It is a non-toxic product and shows limited antimicrobial activity. To improve its efficacy propyl gallate is frequently combined with other antioxidants. The combination of imidazole and propyl gallate gave MIC values 10–150 times lower than those of imidazole alone. Theoretically this combination could reduce the side effects of long treatment with imidazoles and lower the risk of resistance to these antifungal drugs (Strippoli *et al.*, 2000).

Vinyl monomers with phenol and benzoic acid as pendant groups were synthesized, and their antimicrobial activities were examined on bacteria (*Staphylococcus aureus* and *Pseudomonas aeruginosa*) and fungi (*Aspergillus fumigatus* and *Penicillium pinophilum*) using the halo zone test. For both bacteria and fungi, the halo zone diameter decreased in the order of *p*-hydroxyphenyl acrylate > allyl *p*-hydroxyphenyl acetate ≈ *p*-2-propenoxyphenol and polymerization of the monomers decreased their antimicrobial activity significantly (Park *et al*., 2001).

Cinnamic acid derivatives were synthesized and evaluated as antibacterial and antifungal agents. From the results, it is evident that both isobutyl cinnamate and dibromo cinnamic acid exhibited strong antibacterial activity against gram positive (*Staphylococcus aureus*) and gram negative bacteria (*Escherichia coli*) and good antifungal properties on *Candida albicans* and *Aspergillus niger*. The removal of double bond in side chain of cinnamic acid was affected with OH and Br group. The results showed that addition of halogens to the side chain caused remarkable increase in growth inhibitory effect of cinnamic acid whereas addition of hydroxy groups to the side chain double bond did not remarkably enhance the antimicrobial activity (Narasimhan *et al*., 2004).

From these aforementioned studies, data in literature show that benzoic acid derivative and related compound may play a role in the plant disease resistance. Especially, Plant diseases caused by fungi in Thailand are a main loss problem of many kinds of economic crops. Maybe benzoic acid derivatives and related compounds could be utilized to control phytopathogenic fungi of Thailand.

1.3 Objectives of this research

The main objective of this study was to examine the antifungal activity of benzoic acid derivatives and related compounds, in order to define a possible structure–activity relationship.