CHAPTER II

MATERIALS AND METHODS

2.1 Chemicals

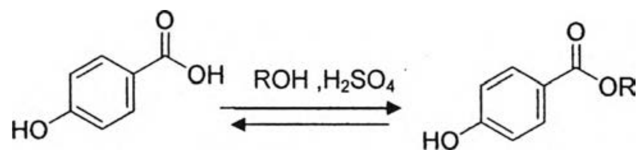
Benzoic acid derivatives, related compounds and Tween80 were purchased from Fluka. Cinnamic acid was purchased from Hopkin&Williams LTD. All of cinnamic acid derivatives were gifts provided by Ms. Sujittra Deesamer (Faculty of Science, Chulalongkorn University). Benzaldehyde was purchased from Baker analyzed. Dimethylsulfoxide (DMSO) was purchased from Merck. Hexyl, octyl and dodecyl 4-hydroxybenzoate were prepared.

2.2 Equipments

Polytetrafluoroethylene (PTFE) membrane 13 mm diameter syringe filter (0.2 μm pore size) was purchased from Chrom Tech. The ^{1}H and ^{13}C-NMR spectra were determined with a nuclear magnetic resonance spectrometer of Varian model Mercury 400. The absorbance value measured using UV-vis spectrophotometer, hp 8543.

2.3 Synthesis of hexyl, octyl and dodecyl 4-hydroxybenzoate

The esters of 4-hydroxybenzoic acid were prepared *via* the reaction of corresponding alcohols in the presence of sulfuric acid. The corresponding alcohol (8 mmol) was poured into a round bottom flask with 4-hydroxybenzoic acid (4 mmol) and sulfuric acid (0.3 ml). The solution was refluxed for 5 h. After completion of reaction, the reaction mixture was washed with saturated $NaHCO_3$ and water. Final product was obtained by purification with column chromatograph.



Hexyl 4-hydroxybenzoate, ^{1}H-NMR ($CDCl_3$) δ (ppm) : 7.93 (d, 2H, J = 8.6 Hz, aromatic protons), 6.91 (d, 2H, J = 8.6 Hz, aromatic protons), 4.31-4.27 (t, 2H, J = 7.02 Hz, -O-CH_2-), 1.78-1.71 (m, 2H, -O-CH_2-CH_2-), 1.44-1.31 (m, 6H, 3x-CH_2-), 0.90-0.87 (m, 3H, -CH_3)

Octyl 4-hydroxybenzoate, ^{1}H-NMR ($CDCl_3$) δ (ppm) : 7.98 (d, 2H, J = 8.58 Hz, aromatic protons), 6.91 (d, 2H, J = 8.6 Hz, aromatic protons), 4.34-4.30 (t, 2H, J = 7.0 Hz, -O-$\mathbf{CH_2}$-), 1.82-1.75 (m, 2H, -O-CH_2-$\mathbf{CH_2}$-), 1.46-1.31 (m, 6H, 3x-$\mathbf{CH_2}$-), 0.93-0.90 (m, 3H, -$\mathbf{CH_3}$)

Dodecyl 4-hydroxybenzoate, ^{1}H-NMR ($CDCl_3$) δ (ppm) : 7.98 (d, 2H, J = 8.6 Hz, aromatic protons), 6.89 (d, 2H, J = 8.6 Hz, aromatic protons), 4.33-4.27 (m, 2H, -O-$\mathbf{CH_2}$-), 1.80-1.76 (m, 2H, -O-CH_2-$\mathbf{CH_2}$-), 1.46-1.31 (m, 6H, 3x-$\mathbf{CH_2}$-), 0.92-0.90 (m, 3H, -$\mathbf{CH_3}$)

2.4 Fungal strains

Four phytopathogenic fungi (*Alternaria porri*, *Fusarium oxysporum*, *Pestalotiopsis* sp. and *Phytophthora parasitica*) were used in antifungal assay. All of these fungal strains was supplied by the Division of Plant Disease and Microbiology, Department of Agriculture, Ministry of Agriculture and Cooperatives, Bangkok, Thailand. The colony characteristics of four phytopathogenic fungi were shown in Fig 2.1. Fungal cultures were grown on potato dextrose agar (PDA) which the formula as presented in appendix A at room temperature. All cultures were maintained and subcultured every month.

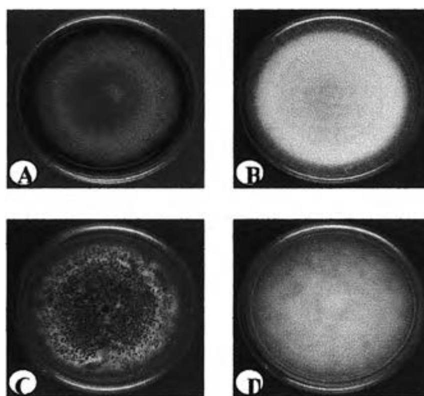


Fig 2.1 The colony characteristics of the four phytopathogenic fungi on PDA used in this research: **(A)** *Alternaria porri*; **(B)** *Fusarium oxysporum*; **(C)** *Pestalotiopsis* sp.; **(D)** *Phytophthora parasitica*

2.5 Antifungal assay

The bioassay was conducted using the agar medium assay (Chavarin, 2002). Benzoic acid derivatives and related compounds have limited solubility in water and thus, for experimental proposes, were dissolved in DMSO (final concentration not exceed 0.4%) in case of solid substance or Tween80 (final concentration not exceed 0.2%) in case of liquid substance. DMSO or Tween80 solutions of tested compounds were applied to a 13 mm diameter PTFE membrane syringe filter (0.2 μm pore size) before being added to sterile PDA (sterilization at 15 psi and 121 °C) to obtain the experimental concentration. Sterile medium (20 ml) was added as aseptically to a 90 mm sterile Petri dish, and a sterile 6 mm diameter cork borer used to remove plugs of mycelium from the edges of stock cultures, which were inverted and placed in the center of each Petri dish. Control plates contained cultures medium plus 0.4% DMSO or 0.2% tween80. The inoculated plates were incubated at room temperature. Redial measurements of growth were taken when fungi reached the edge of the control plate, colony diameter was measured in centimeters and calculated for percent inhibition. Each test was replicated three times, and the data was averaged. The IC_{50} values (the concentration in mM that inhibited 50% of the mycelium of fungi growth) were calculated by probit analysis (Cheng *et al*., 2005). Captan and Iprodione, commercially available fungicides were employed as the reference compounds. The formula of percentage inhibition was shown as follows:

$$\text{Percentage inhibition} = \frac{(C - T) \times 100}{C}$$

C : colony diameter of control plate (cm)

T : colony diameter of treatment (cm)

2.6 Spore germination assays

The tests were performed according to Lattanzio *et al.* (1996) with some modification. DMSO solutions of compounds, sterilised by filtration through a 13 mm diameter PTFE membrane syringe filter (0.2 μm pore size), were added to autoclaved PDA after it had cooled to 50°C. Spores obtained from 7-day-old cultures of *Pestalotiopsis* sp. maintained on PDA plates were used to prepare suspensions in sterile distilled water.

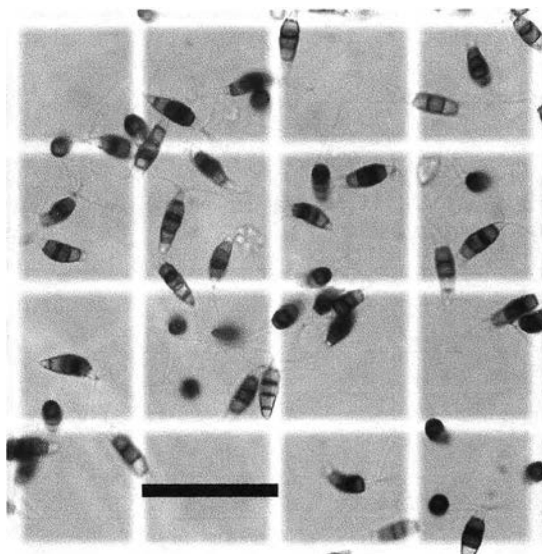


Fig 2.2 Microscopic field of haemocytometer slide with spores of *Pestalotiopsis* sp.; bar = 50 μm

The concentration of spores per ml was estimated with haemocytometer slide (depth 0.1 mm, 1/400 mm^2) under microscope (Fig 2.2). To test spore germination, 100 μl of the suspensions (5 × 10^3 spore per ml) were streaked aseptically on PDA plates, 100 mm in diameter, supplemented with different compound concentrations (0, 0.1, 0.5 and 1 mM). The plates were incubated at room temperature for 48 h. Following incubation, fungal colonies originated from germinated spores were enumerated in order to evaluate the %inhibition of spore germination, which were calculated as follows:

$$\%\text{inhibition of spore germination} = \frac{(C - T) \times 100}{C}$$

C : mean fungal colony of control plate

T : mean fungal colony of treatment

2.7 Phytotoxicity bioassays

Bioassays used Petri dishes (90 mm diameter) with one sheet of Whatman No.1 filter paper as substrate. Germination and growth were conducted in aqueous solutions. Solutions of the compounds to be assayed were prepared in DMSO and

then diluted to reach the test concentrations (0.05 mM and 0.1 mM). The number of seeds in each Preti dish was 20 and 5 mL of treatment, control (0.1% DMSO) or compound solution was added to each Petri dish. The plates were incubated at room temperature in the dark. Three replicates were used (60 seeds). Seed germination and root length in each plate were measured at 4 days. The percentages of relative root growth were calculated as follows:

$$\text{Relative root growth (\%)} = \frac{\text{mean root length in solution of compound}}{\text{mean root length in control}} \times 100$$

2.8 Stability test of 2,6-dichlorocinnamic acid

The 2,6-dichlorocinnamic acid were evaluated under oven conditions at 80 °C, daylight and 256 wavelength UV light for a period of 8 h. For each condition, 2,6-dichlorocinnamic acid were stored in small bottles. Samples were with drawn at 0, 2, 4, 8 h from the test conditions, placed in test tubes, capped and stored in a dessicator until analyzed. DMSO solutions of each collected sample were prepared at concentration of 0.5 mM. The solutions of 2,6-dichlorocinnamic acid were analyzed for stability by using a UV-Vis spectrophotometer. Absorbance was measured at lamda max and compared among each collected time.

2.9 Statistical analysis

For each of the data collected, relative standard deviations are given. Probit analysis and *t*-test were then performed. All computations were done using the SPSS program.