CHAPTER 3

MATERIALS AND METHODS

Fungal isolation and culture

Three fungi, *Pestalotiopsis* sp., *Phomopsis* sp. and *Curvularia* sp., were obtained from the Isolation Collection of the Plant Protection Institute, Department of Agriculture, Ministry of Agriculture and Co-operative, Thailand. *Lasiodiplodia theobromae* (Pat.) Griffon and Maubl. strain LP20, the most virulent strain from a pathogenicity test and isolated from infected longan fruit, was obtained from the Department of Biology, Faculty of Science, Chiang Mai University, Thailand. They were cultured on potato dextrose agar (PDA) (Sigma, St. Louis, MO, USA) (Rasrinaul, 1996).

Longan

Longan fruit cv. Daw was purchased during the main harvesting season from a commercial orchard in Chiang Mai province. They were transported to the laboratory within 2 h after harvest. Fruit were selected for uniformity in size. Fruit were prepared by cutting the stem to a 0.5 cm in length. *L. theobromae*-inoculated fruit were prepared by placing 5 mm diameter mycelial discs, taken from the periphery of two-day old active cultures, on the pericarp of the stem-end of longan fruit, then incubating the fruit in a closed plastic box with a moist tissue for 6 h at ambient temperature. The mycelia disc was then removed from the fruit before further use.

Preliminary study: Effect of five volatile compounds on inhibition of mycelium growth of four postharvest decay fungi of longan fruit *in vitro*

A mycelial disc (5 mm diameter) was taken from the periphery of actively growing fungal cultures and placed at the center of a 90 mm x 15 mm Petri dish (92.5 ml) containing 20 ml of PDA with a sterile cap placed to one side. The agar disc was

inverted so that the inoculum was in direct contact with the agar. Different volumes of each volatile compound were placed in the sterile cap inside each dish. *Trans*-2-hexenal, (purity ≥95%, Sigma, St. Louis, MO, USA) was placed in the sterile cup inside each dish to achieve concentrations of 0 (control), 33, 66, 132, 198 and 264 µl l^{-1}, based on an air volume of 75 ml per dish. For hexanal, 2-nonanone, methyl benzoate, and methyl salicylate (purity ≥98%, ≥99%, ≥99% and ≥99%, respectively; Sigma, St. Louis, MO, USA) were placed in the sterile cup inside each dish to achieve concentrations of 0 (control), 66, 132, 198, 264, 396 and 660 µl l^{-1}, based on an air volume of 75 ml per dish.. The control treatment consisted of sterile distilled water instead of volatile compound. The dishes were quickly sealed with Parafilm and incubated at 25°C in the dark. There were five replicates of each concentration of each volatile for each fungus.

The diameter of the colonies was recorded daily until control mycelia were close to 90-mm diameter. This required 3 days for *L. theobromae*, 7 days for *Pestalotiopsis* sp. and *Phomopsis* sp., and 10 days for *Curvularia* sp. The inhibitory effect of each volatile treatment on mycelial growth was calculated as:

$$\text{Inhibitory rate on mycelia growth (\%)} = \frac{(\text{Control colony diameter} - \text{Treatment Colony diameter}) \times 100}{\text{Control colony diameter.}}$$

Experiment 3.1 Effect of hexanal on growth and morphology of mycelium, germination and morphology of spores, and activity of four cell wall degrading enzymes of *L. theobromae.*

3.1.1 Effect of fumigation time on mycelium growth, spore germination and morphology of *L. theobromae in vitro*

Hexanal was chosen for subsequent study based on antifungal activity, reported below, and because it is commercially available at a reasonable cost in Thailand, and it is approved as a food additive by the U.S. Food and Drug Administration, and has an ORL-MAM LD50 of 3700 mg kg^{-1} (EAFUS, 2006).

Seven volumes of hexanal, 0, 66, 132, 198, 300, 600 or 900 µl l^{-1}, were used to treat mycelia of *L. theobromae* for 1, 2, 24 or 48 h. The exposure methods were the same as described above, with the cups containing hexanal removed from the plates after the designated intervals, and the plates closed and re-sealed. If mycelial growth was not observed by the time control mycelia reached 90 mm, the disc was removed from the dish and placed in a new Petri dish containing PDA but no hexanal to evaluate whether the hexanal effect was either fungistatic or fungicidal. A compound was considered as fungistatic if mycelia grew during the additional incubation period, or fungicidal if mycelial growth was not detected during this period. The experiment was repeated twice.

L. theobromae did not produce spores on PDA medium under this study's culture conditions, so spore preparation and fumigation were performed as follows. A 5 mm diameter mycelial disc of *L. theobromae* was used to inoculate the surface of mangosteen (*Garcinia mangostana*) fruit after sterilization with 70% ethyl alcohol.

The inoculated fruit were incubated in a closed plastic box with moist tissue paper for 24 h, and then were transferred to room temperature (~ 30°C) until sporulation was observed. A brush was used to remove the spores from the mangosteen fruit surface. The spores were kept in a closed bottle in a refrigerator until used (~ 3°C).

A spore suspension in sterile distilled water, filtered through sterile cloth, was prepared at a spore density of 10^{-6} conidia ml^{-1} using a haemacytometer. A 100 µl aliquot of spore suspension were spread on a 9-cm diameter Petri dish containing 20 ml PDA with a sterile cup to one side. Volatile compounds were added to the sterile cup as described above from 0 to 900 µl l^{-1}. The dishes were sealed with Para film and incubated at 25°C. The cups were removed from the dishes after 1, 2, 24 and 48 h. Three replicate plates per hexanal concentration by duration of exposure were taken to count spore germination under a compound microscope (CX31-12L02-set, Olympus, Japan) daily, with germination indicated when the germ tube extended more than the spore diameter, and for observing spore morphology under a compound microscope (Nomarski DIC model DP 12-PG, Olympus, Japan) after fumigation for 6

and 12 h which were the periods that the normal spores would be germinated. The plates exhibiting ungerminated spores were incubated for 3 additional days, and the effect was considered fungistatic when the spores germinated and fungicidal when the spores did not germinate. The experiment was repeated twice.

3.1.2 Effect of hexanal on mycelium morphology of *L. theobromae* on PDA

L. theobromae mycelia that had been grown for 3, 7 or 14 days on PDA were fumigated with 0, 478, 956 and 1,434 µl l^{-1} of hexanal, for 24 h as described above. After 24 h, the mycelial morphology was observed using a compound microscope (Nomarski DIC model DP 12-PG, Olympus, Japan).

3.1.3 Effect of hexanal on extracellular enzyme activities of *L. theobromae* on PDA

Mycelial discs (5 mm) of *L. theobromae* were taken from the periphery of an actively-growing culture and placed in 90 mm Petri dishes containing 20 ml PDA for 14 days at 25°C before fumigation with hexanal at 0, 478, 956 and 1,434 µl l^{-1} for 24 h at 25°C, with sterile distilled water as the control. There were four replications of each hexanal concentration with fifteen Petri dish cultures in each replication.

After hexanal fumigation, fungal mycelia were washed to remove extracellular protein by gentle pouring and collection of ice-chilled water (10ml) four times across the PDA medium (Myung *et al*., 2007). Each mycelial wash was shaken at 100 rpm for 120 sec, 30 sec rotation per direction, before pouring off. In each replication, fungal wash samples from the 15 plates were combined. Then the fungal wash samples were passed through Miracloth and stored at -80°C (Deep Freezer -86°C, MDF-U71V, Sanyo, Japan).

The frozen fungal wash samples were lyophilized (Flexi-Dry MP model, Kinetics, USA), then resuspended in 5 ml of 4°C Millipore-purified water. The suspension was centrifuged at 15,000 rpm at 4°C for 20 min. The supernatant was re-frozen and held at -80°C prior to determining the activity of four enzymes:

cellulase, polygalacturonase (PG), pectin methylesterase (PME) and cutinase. Final protein content of the supernatant was determined by the method of Bradford (1976).

3.1.3.1 Cellulase assay

Cellulase activity was determined by measuring the reducing groups released from carboxymethyl cellulose. The concentrations of reducing groups were determined using D-glucose as a standard. The reaction mixture consisted of 100 µl of fungal wash solution and 900 µl of 1% carboxymethyl cellulose in 0.05 M of sodium citrate buffer at pH 4.8. Incubation was carried out at 37°C for 1, 2, 3 and 4 h. After incubation, 1 ml of PGO enzyme color reagent (Sigma) was added to 100 µl of reaction mixture and incubated at 37°C for 30 min. The absorbance was read at 450 nm (UNICAM UV 500 UV/VIS spectrophotometer, ThermoSpectronic, Cambridge, UK). Reaction solution without substrate was used as control. Cellulase activity was expressed as µmol glucose produced per h per mg protein (µmol glucose$\cdot h^{-1} \cdot mg^{-1}$ protein).

3.1.3.2 Polygalacturonase assay

Polygalacturonase activity was determined by measuring the reducing groups released from polygalacturonic acid (Sigma). Reducing groups were quantified with 3, 5-dinitrosalicylic acid with slight modification of the method described by Wang, *et al*. (1997). The reaction mixtures, containing 1 ml of 0.5% polygalacturonic acid, 1 ml of 0.1 M sodium acetate buffer (pH 4.5), 100 µl of fungal wash, and 900 µl of deionized water, were incubated at 30 ± 2°C for 50 min. After incubation, 1 ml of 3, 5-dinitrosalicylic acid solution was added and boiled at 95 ± 3 °C for 10 min. The change in absorbance was recorded at 575 nm (A575). PG activity was expressed as the increase in A575$\cdot min^{-1} \cdot mg^{-1}$ protein.

3.1.3.3 Pectin methylesterase assay

Pectin methylesterase (PME) activity was determined by the method of Hagerman and Austin (1986). All solutions, pectin, bromothymol blue, water and fungal wash were adjusted to pH 7.5 before starting. Reaction mixtures consisted of a 0.01% (w/v) solution of bromothymol blue prepared in 3 mM potassium phosphate buffer at pH 7.5. Solutions of 0.5 % (w/v) citrus pectin were prepared in distilled water and adjusted to pH 7.5 with concentrated NaOH. In a cuvette, 2 ml of pectin

was mixed with 150 µl of bromothymol blue, 650 µl of water, and 200 µl of fungal wash. The decrease in absorbance at 620 nm (A620) of the mixture was determined versus water for 20 min. PME activity was expressed as the decrease in A620·min^{-1}·mg^{-1} protein.

3.1.3.4 Cutinase assay

Cutinase activity was determined by a modification of the method of Macedo and Pio (2005), following the hydrolysis of *p*-nitrophenylbutyrate (PNPB). A 20 µl aliquot of the fungal wash was added to 700 µl of a reaction mixture having the following composition: 0.56 mM PNPB dissolved in 50 mM phosphate buffer at pH 7.0, containing 0.2% Triton X-100 and 0.43 M tetrahydrofuran. The reaction was monitored for 10 min at an absorbance of 405 nm (A405) against a blank solution. Cutinase activity was expressed as the increase in A405·min^{-1}·mg^{-1} protein.

Experiment 3.2 Effect of hexanal on postharvest decay of longan fruit

3.2.1 Influence of hexanal concentration and fumigation time on longan fruit decay

Fruit were fumigated with hexanal vapor by placing ten fruit on wire mesh in an 850 ml glass bottle with a sterilized filter paper. A volume of hexanal was quickly added to the filter paper, and the bottle was sealed with a locking lid for a designated period of time. For controls, a volume of sterilized distilled water was added equal to that of hexanal. After treatment, fruit were transferred to a foam tray and wrapped with translucent PVC plastic film. The trays were placed in 5^{o}C storage for up to 30 days.

Four hexanal concentrations, 0, 300, 600 and 900 µl l^{-1}, were used to treat *L. theobromae*-inoculated and non-inoculated fruit for five durations, 0, 1, 2, 3, 4 or 6 h, at ambient temperature. There were four replicated bottles of 10 fruit each per concentration by duration of treatment. The appearance of fungi on the fruit and severity of decay were recorded by rating fruit at 5 days intervals during cold storage. The percentage of fruit decay was recorded on each date, and severity of fungal development on the surface was rated from 0 to 4 with 0 = no visual evidence of

fungi, 1 = less than 10%, 2 = 10-30%, 3 = 31-70%, and 4 = more than 70% of the surface affected with fungus. Mean scores of fungal development were calculated as:

Fruit decay (%) was calculated as: $\frac{\Sigma \text{ ((number of fruit in fungal score 1-4) X 100)}}{\text{Total observed fruit.}}$

Fungal incidence scores and phytotoxicity were calculated as:

$$\frac{\Sigma \text{ (number of fruit in each score X score)}}{\text{Total observed fruit.}}$$

3.2.2 Influence of hexanal fumigation temperature on longan fruit decay

Because hexanal at 900 µl l^{-1} for 2 h seemed to be the best treatment for least fruit decay from Exp. 3.2.1, it was chosen to fumigate non-inoculated and inoculated fruit at 3 temperatures, ambient temperature, 40 and 5°C, for 2 h. After fumigation, fruit were stored and evaluated as in Exp. 3.2.1.

3.2.3 Hexanal metabolism by longan fruit

Ten longan fruit were fumigated with hexanal at 900 µl l^{-1} in an 850 ml closed bottle with a gas septum on the fitted lid for 24 h at ambient temperature. Then, 100 µl headspace vapor samples were trapped using a 100 µM PDMS of SPME syringe for 30 min. The sample was analyzed by GC-MS (Agilent Technologies GC 6890) using a Hewlett Packard instrument with a 30 m x 0.25 mm ID x 0.25 µm HP-5MS column. The injector and FID detector temperatures were 250°C. Helium was used as the carrier gas at flow rate of 1.0 ml/min. The oven temperature was 40°C for 5 min, and then raised 10°C /min to 230°C where it was held for 6 min, for a total runtime of 30 min. Headspaces from bottles with only hexanal and only longan fruit were also analyzed as above to identify hexanal metabolites in fumigated longan fruit. This work was done by the Science and Technology Service Center, Chiang Mai University. The study was repeated three times.

Experiment 3.3 Effect of hexanal on longan fruit quality

Due to the browning observed in longan fruit after hexanal fumigation, a Sta-fresh wax coating was used to reduce or eliminate the problem. Non-inoculated fruit were dipped for 1 min in 5% (v/v) Sta-fresh 310 wax coating (FMC International, UK) at ambient temperature. After dipping, the fruit were air-dried for 30 min before exposure to hexanal at 900 $\mu l \cdot l^{-1}$ for 2 h at ambient temperature as above. The coating plus hexanal treatment was compared to no treatment, coating alone, and hexanal alone. There were multiple replicate bottles of 10 fruit each per treatment, enough to provide fruit for the destructive sampling on each sampling date as described below. Samples were taken randomly every 3 d to determine weight loss, juice total soluble solids, pH, pericarp color, pericarp electrolyte leakage, fungal incidence, fruit decay and hexanal residue in pericarp and aril as described below. Consumer acceptance was evaluated every 5 days. Colony forming units (CFU) of microorganisms in pericarp and aril were determined every 6 days. The experiment was terminated at 24 d due to loss of control fruit to disease.

3.3.1 Determination of weight loss

Weight of ten fruit per treatment was determined by removing the fruit from storage, individually weighing each on a balance (± 0.01 g) Model PB1502-S (Mettler-Toledo, Menlo Park, CA, USA), sampling the fruit in each treatment every three days intervals.

3.3.2 Measurement of total soluble solids content

Arils of five fruit per treatment on each sampling date were individually macerated in a blender and the puree was filtered through cheesecloth before measurement. TSS of the filtered juice was determined using a digital refractometer (Pocket PAL-1, Japan). Values were reported as % brix.

3.3.3 Measurement of pH

Using aril samples from 3.3.2 above, the pH of the juice was measured using a pH meter (Consort C831, Belgium). Five fruit pericarp were ground to a powder in a Molinex blender. Ground sample (2 g) was homogenized with 20 ml distilled water before measurement.

3.3.4 Determination of electrolyte leakage

Pericarp electrolyte leakage was determined by the method of King and Ludford (1983) with some modifications. Pericarp discs (0.5 cm diameter) were collected from each of 10 replicate fruit per treatment by sampling date, rinsed 3 times in Millipore-purified water, and soaked in 25 ml of 0.4 M mannitol solution at ambient temperature for 3 h. Initial electrical conductivity readings were determined using a conductivity meter (Sartorius Professional Meter PP-20, Sartorius AG, Germany). After the initial reading, the flasks were autoclaved (HL-341, Gemmy Industrial Corp., Taiwan, Speedy Autoclave vertical type) at 121^{o}C and 15 PSI for 30 min. After cooling to ambient temperature, the final conductivity reading was determined for total electrolytes. Percent electrolyte leakage was calculated as the ratio of the initial reading to the final reading as:

Electrolyte leakage (%) = (initial conductivity/total conductivity) X 100.

3.3.5 Measurement of firmness

Firmness was measured as the maximum force required to penetrate into the aril using a Texture Analyzer TA XT plus (Texture Analyzer Corp., England) with a 50 kg load cell. A 2 mm diameter flat head stainless steel cylindrical probe was set to penetrate 20 mm into the fruit at a speed of 0.1 mm/min. Five fruits were measured for each treatment by sampling date.

3.3.6 Measurement of the pericarp and aril color

The color values, brightness (L*); chroma (C); and hue angle (H) of outer and inner pericarp surfaces were measured on five replicate fruit of each treatment on each sampling date using a Hunterlab color meter (Color Quest XE, The

Color Management Company, Reston, Virginia, USA). Measurements were taken on two opposite sides of five fruit for each sample and averaged for each fruit.

3.3.7 Analysis for hexanal residue in pericarp and aril

The hexanal residue content of pericarp and aril tissues of longan fruit was determined by gas chromatographic analysis according to a method developed by Barrett *et al*. (2000) with some modification. Five replicate fruit per treatment on each sampling date were used. One g of the pericarp or 3 g of aril tissue was placed in a 10 ml glass bottle which was closed with a rubber cap and incubated in a water bath at 60^{o}C for 30 min. Then, 100 µl of headspace gas was withdrawn and injected into a TRACE gas chromatograph (ThermoQuest Italia SpA, Italy) equipped with a flame ionization detector. The temperature of the oven, injector and detector were 40, 230 and 230^{o}C, respectively. The column used was a 30 m x 0.53 mm i.d. x 1 µm OV-1 (100% dimethylpolysiloxane) capillary column. Retention time and a standard curve of absolute hexanal in water solution (0 to 100 µg/g) were determined.

3.3.8 Detection of colony forming units (CFU) of microorganisms isolated from the pericarp and aril

Five fruit from each treatment by sampling date were immersed in 50 ml of 0.1% sterilized peptone and shaken for 30 min. Then, the solution was diluted by 10^{-1} or 10^{-2} with 0.1% sterilized peptone, 100 µl of each concentration were spread over PDA medium and incubated at 25^{o}C for 3 days before counting total CFU. CFU from aril tissue was done as with pericarp, but after the fruit were sterilized by soaking in 90% ethanol for 1 min, 2.5% sodium hypochlorite for 3 min and 95% ethanol for 30 sec before removing the pericarp (Johnson *et al.*, 1989). Data was expressed as the mean number of colony forming units per ml (CFU ml^{-1}).

3.3.9 Evaluation of fruit quality acceptance

Fruit aroma, flavor and overall quality acceptance of outer pericarp, inner pericarp, and aril were evaluated by scoring each from 1-5. Five well-trained evaluators scored each fruit as 1= mostly dislike, 2 = moderately dislike, 3= neither

like nor dislike, 4 = moderately like and 5= mostly like. Panelists were given one fruit per treatment from each sampling date.

3.3.10 Evaluation of fruit decay and fungal incidence

Fruit decay and fungal incidence were evaluated as in 3.2.1 above.

Experiment 3.4 Effect of hexanal on some chemical components and some biochemical characteristics of longan fruit pericarp inoculated with *L. theobromae*

Hexanal was used to fumigate non-inoculated fruit and *L. theobromae*-inoculated fruit at 0 or 900 µl l^{-1} for 2 h at ambient temperature. After fumigation, fruit were stored at 5°C, 10 fruit per foam tray wrapped with PVC plastic. Samples from each treatment were removed from cold storage every 3 days. Longan pericarp from ten fruit per treatment were removed, frozen in liquid nitrogen and ground to a powder using a Molinex blender. The ground samples were held at -80°C before determination of phenolic compound content, and activities of polyphenoloxidase (PPO), peroxidase (POD), polygalacturonase (PG), and cellulase. The protein content was determined according to Bradford (1976).

3.4.1 Determination of phenolic compounds

3.4.1.1 Extraction of phenolic compounds

Phenolic compounds were extracted according to the methods of Zhang *et al*. (1999) and Sun *et al*. (2002) with slight modification. One g of ground sample was homogenized in 10 ml cold ethanol (80%) and centrifuged at 4,010 x g for 30 min at 4°C. The supernatant was collected and kept at -80°C before determination.

3.4.1.2 Determination of total phenolic content

The total phenolic content was determined by placing 100 µl of supernatant and 900 µl of water in test tubes followed by addition of 5 ml of 10% Folin-Ciocalteu reagent (Dewanto *et al*., 2002). The samples were mixed well and left for 8 min. Then, 4 ml of a 7.5% sodium carbonate solution was added and the

samples held for 2 h at room temperature. The absorbance was measured at 765 nm versus a blank. Phenolic compound content was expressed as mg of gallic acid equivalents, derived from a standard curve of gallic acid, per g fresh weight. Each replicate sample was assayed twice, and the mean of the two assays for each replicate was used for statistical analyses.

3.4.2 Determination of polyphenoloxidase activity (PPO) in pericarp

3.4.2.1 Extraction of polyphenoloxidase (PPO)

PPO was extracted according to the methods of Jiang (1999) with some modification. One gm of ground sample was homogenized with 10 ml of 0.1 M sodium phosphate buffer (pH 6.4) containing 1% polyvinylpyrrolidone (PVP), and was then held overnight at 4°C. The homogenate was subsequently centrifuged at 4,010 x g for 30 min at 4°C. The supernatant was collected and stored at -80°C before assay.

3.4.2.2 PPO activity assay

PPO activity was assayed with pyrocatechol as a substrate by a spectrophotometric procedure. A 50 µl aliquot of the crude supernatant was added to 3 ml of a reaction mixture consisting of 0.1 M pyrocatechol in 0.1 M sodium phosphate buffer at pH 6.4. The increase in absorbance at 25°C was recorded continuously at 400 nm for 3 min. The protein content was determined according to Bradford (1976). One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.01 absorbance units per minute per mg protein. Each replicate sample was assayed three times, and the mean of the three assays for each replicate was used for statistical analyses.

3.4.3 Determination of peroxidase activity

3.4.3.1 Extraction of peroxidase

Peroxidase was extracted according to the methods of El-Hilali *et al.* (2003) with some modification. One gram of ground pericarp was homogenized in 10 ml of 0.05 M sodium phosphate buffer with 0.1 M NaCl (pH 6.4). The extract was

centrifuged at 4,010 x g for 30 min at 4°C. The supernatant was collected and kept at -80°C before assay.

3.4.3.2 Peroxidase activity assay

POD activity was assayed with guaiacol as a substrate by a spectrophotometric procedure (Zhang *et al*., 2005). An aliquot of 20 µl of the crude supernatant was added to 3 ml of a reaction mixture consisted of 0.1% H_2O_2 and 0.5% guaiacol in 0.01 sodium acetate buffer (pH 6.0). The increase in absorbance at 470 nm was recorded continuously for 3 min at 25°C. One unit of enzyme activity was defined as the amount of the enzyme which caused a change of 0.01 in absorbance unit per minute per mg protein. Each replicate sample was assayed three times, and the mean of the three assays for each replicate was used for statistical analyses.

3.4.4 Determination of polygalacturonase and cellulose

3.4.4.1 Extraction of polygalacturonase and cellulase

PG and cellulase were extracted according to the methods of Peng *et al.* (2004) with some modifications. Three grams of a ground sample was homogenized in 10 ml of 0.1 M phosphate buffer at pH 6.4 containing 0.1M ascorbic acid, 5% insoluble polyvinylpyrrolidone (PVPP), and 1mM EDTA. Then, the homogenate was filtered through two layers of cheesecloth and centrifuged at 12,000 x g for 20 min at 4°C. The supernatants were collected and kept at -80°C for assay

3.4.4.2 Polygalacturonase assay

Polygalacturonase (PG) activity was determined by measuring the reducing groups released from polygalacturonic acid (Sigma). Reducing groups were measured with 3, 5-dinitrosalicylic acid with slight modification of the method described by Peng (2004). The reaction mixture, containing 1 ml of 0.5% polygalacturonic acid, 1 mL of 0.1M sodium acetate buffer (pH 4.5), 50 µl of extract supernatant, and 950 µl of 0.1M phosphate buffer (pH 6.4) were incubated at 30 ± 2 °C for 50 min. Samples were taken every 10 min and 1 ml of 3, 5-dinitrosalicylic acid solution was added, and the mixture was boiled at 95 ± 3°C for 10 min. The absorbance (A) was recorded at 575 nm. Polygalacturonase activity was determined

by measuring the reducing groups released from polygalacturonic acid and reported as A575·min^{-1}·mg^{-1} protein.

3.4.4.3 Cellulase assay

Cellulase activity was determined by measuring the reducing groups released from carboxymethyl cellulose. Reducing groups were measured with 3, 5-dinitrosalicylic acid with slight modification of the method described by Wang *et al*. (1997). The reaction mixture, containing 1 ml of 0.5%carboxymethyl cellulose, 1 ml of 0.1M sodium acetate buffer at pH 4.5, 50 µl of extract supernatant, and 950 µl of 0.1M phosphate buffer at pH 6.4, were incubated at 30 ± 2^{o}C for 50 min. The sample were taken every 10 min to add 1 ml of 3, 5-dinitrosalicylic acid solution and boiled at 95 ± 3^{o}C for 10 min. The absorbance (A) was recorded at 575 nm. Cellulase activity was expressed in absorbance per min per mg protein (A575·min^{-1}·mg^{-1} protein).

Statistical analysis

All statistical analyses were performed by analysis of variance (ANOVA). Percent inhibition data were transformed to arcsine values before analysis. Mean comparisons were determined by the least significant difference (LSD) test at a significant level of $P = 0.05$.