CHAPTER 3

Materials and Methods

3.1 Materials

3.1.1 Plant materials

A number of immature Nam Dok Mai mango fruits were purchased from a company exporting mango (Shine Forth Co. Ltd, Bangkok, Thailand). Fruits were packed in carton boxes, transported to the laboratory and stored in a closed refrigerated chamber at 4-5°C until use (maximum 3 days of storage). Mangoes which had uniform size and appearance without any mechanical injuries and disease symptoms were selected for the experiments.

3.1.2 Chemicals and pathogen cultures

Sodium hydroxide (NaOH), phenolphthalein, 2,6 dichlorophenolindophenol, meta-phosphoric acid (HPO_3), acetic acid (HOAc), sodium bicarbonate ($NaHCO_3$), L-ascorbic acid, acetone, petroleum ether, anhydrous sodium (Na_2SO_4) sulfate, β-carotene, ethanol (99.9 %), sodium carbonate (Na_2CO_3), Foline-Ciocalteu reagent, Gallic acid monohydrate, magnesium sulfate ($MgSO_4$), sodium acetate (NaOAc), acetonitrile (MeCN), acid acetic (HOAc), Tween 20, deionized (DI) water, potassium iodile, hydrogen peroxide, hydrochloric acid (HCl) and toluidine blue were of analytical grade and purchased from Sigma- Aldrich (St Louis, MO, USA), Loba Chemie (Rd Wodehouse, Mumbai, India) and ACI Labscan (Rd Pathumwan, Bangkok, Thailand).

Analytical standard pesticides chloropyriphos (O,O-diethyl O-3,5,6-trichloro-2-pyridyl phosphorothioate), cypermethrin ((RS)-alpha-cyano-3 phenoxybenzyl (1RS)-cis-trans-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylate), carbendazim (Methyl N-(1H-benzoimidazol-2-yl) carbamate) were purchased from Dr. Ehrenstorfer GmbH (Augsburg, Germany).

Potato dextrose agar (PDA) and streptomycin sulfate powder were purchased from HiMedia (Marg, Mumbai, India) and Sigma-Aldrich (St Louis, MO, USA).

Colletotrichum gloeosporioides was supplied from Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai, Thailand as the test pathogen. Subculture and reisolations of the fungus were done in PDA for the experiments.

3.2 Effect of NTP on decontamination of pesticide residues covered on mango and physicochemical as well as sensory attributes of the fruit

3.2.1 Covering pesticides (chlorpyrifos, cypermethrin and carbendazim) in mango fruits

Chlorpyrifos and cypermethrin solutions were prepared separately by adding 0.02 g of the standard pesticide powder into 200 mL of acetone nitrile (MeCN) containing Tween 20 (1 %) as a surfactant which help to adhere more pesticide to the smooth surface of mango skin. Then each pesticide solution (100 ppm) was sprayed 4 times onto both sides of mango fruits (10 mL per fruit) under the fume hood and the fruits sprayed with pesticide solution were dried by air flow from the electric fan.

For covering mango with carbendazim, mangos were immersed totally in the pesticide solution (200 ppm – 200 mg of carbendazim in 1 L of MeCN containing 1 % of Tween 20) for 15 min (10 fruits per 1000 mL of carbendazim solution). The dipped pesticide mangos were allowed air-dry under fume hood for 5 min, followed by a second dip for 15 min and repeated drying.

3.2.2 NTP treatment

In this study, the NTP system was developed from Kim *et al*. (2013) using gliding arc (GA) discharge with four major parts including (1) GA discharge system driven by neutron power supply; (2) gas-transport system to provide controlled flows of Argon (Ar) gas and vapor of micro-bubble water to the GA discharge system; (3) control devices; (4) a reservoir tank containing distilled water as shown in Figure 3.1. The basic approach in this NTP design was to have both Ar gas and vapor of distilled water under micro-bubble form pass through the GA discharge system first and then to introduce

plasma-treated Ar gas and water vapor to a large volume of water at the reservoir to decontaminate pesticide residues covered on mango.

The GA discharge was chosen to generate NTP in this study due to its unique plasma properties and the gas flow inside the GA generator creating a sufficiently high degree of non-equilibrium to sustain the selective process. Firstly, the arc discharge would ignite as thermal plasma at the point of the smallest gap between two electrodes, then, the arc would be forced to move downstream by the gas flow and be convectively cooled by a stream of gas which is at room temperature to become a non-equilibrium or non-thermal plasma. Water under micro-bubble form offered the slower rate of rise to the water surface and escaping to the atmosphere because of the reduced buoyancy, accordingly increasing residence time and probability that plasma active species will make contact with the targets (Kim *et al*., 2013).

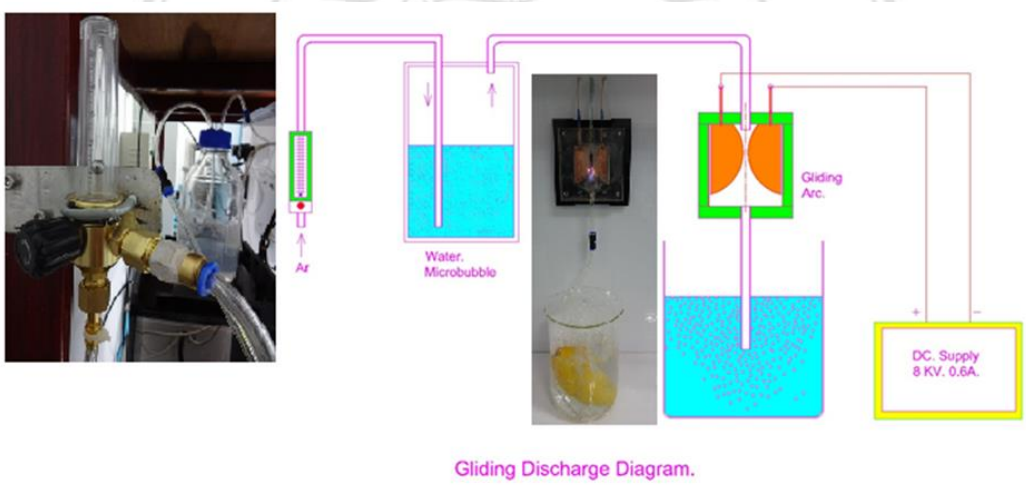

Figure 3.1 Schematic of NTP system treated Nam Dok Mai mango covered pesticide

Micro-bubble water vapor used for generating plasma was produced by applying electro-flotation method with the DC generator (IDP 1010S). Three liters of distilled water containing 1.5 g sodium bicarbonate ($NaHCO_3$) (approximately 0.05 % of concentration) were added in the chamber, then the two electrodes (anode and cathode) were inserted with the 1.5 cm of gap distance and the DC power (180 V and 1.4 A) was supplied for 30 min to form micro-bubble solution (Figure 3.2). There was 500 mL of micro-bubble water was used to generate NTP for each treatment.

For each treatment, one mango fruit covered with the pesticide was put into the glass beaker (reservoir) containing 1 L of distilled water, then the vapor mixture of Ar

gas and micro bubble water passed through the GA discharge supplied by neutron generator and DC transformer (8 kV and 0.6 A) to generate plasma at 600 W, after that cold plasma-treated Ar gas and water vapor were transferred into the beaker. Temperature of all treatments was at room temperature (28^{o}C). There were two varied factors which were the flow rate of Ar gas from 2 L/min to 8 L/min and the time treatment in the range of 1 to 15 min.

Figure 3.2 Micro bubbles generation by electro-flotation method

NTP treatment conditions for decontamination three different pesticide residues (chlorpyrifos, cypermethrin or carbendazim) covered in mango surface were coded and showed in Table 3.1. There were two replications manipulated for each NTP treatment.

After the NTP treatment, the mango fruit samples were recovered and taken for chemical residue measurement. The control was the pesticide covered fruit soaked in distilled water for 15 min (without any NTP treatment). The original quantity of each pesticide residue attached to mango surface by spraying or dipping was evaluated also and coded as the original.

Measurement of $OH^{\bullet}$ radicals emission signal and concentration of hydrogen peroxide (H_2O_2) was done after NTP treatment. Optical emission spectroscopy (OES) equipped with the AvaSpecULS2048CL-EVO (CMOS linear image sensor detector) possessing wavelength range of 200-1100 nm (Avantes, USA) was employed to determine the signal emission at 310 nm of $OH^{\bullet}$ radicals generated according to NTP conditions (Table 3.1).

Table 3.1 Conditions and codes of different plasma treatments for decontamination of pesticide residues covered in mango fruits

Test ID	Flow rate of Ar gas (L/min)	NTP treatment time (min)
Original	Mango covered the pesticide without any treatment	
Control	Mango fruits were only soaked in distilled water for 15 min	
2L_1min	2	1
2L_5min	2	5
2L_10min	2	10
2L_15min	2	15
5L_1min	5	1
5L_5min	5	5
5L_10min	5	10
5L_15min	5	15
8L_1min	8	1
8L_5min	8	5
8L_10min	8	10
8L_15min	8	15

Besides measuring $OH^{\bullet}$ radicals, hydrogen peroxide (H_2O_2) amount produced by NTP conditions (Table 3.1) was also evaluated, because a major pathway for H_2O_2 is the hydroxyl radical recombination in the plasma channels (Banaschik *et al*., 2015; 2016):

$$OH^{\bullet} + OH^{\bullet} \rightarrow H_2O_2$$

Hydrogen peroxide concentration was measured following to spectrophotometric determination method of Sunil and Narayana (2008). Firstly, 3 mL of NTP treated samples was added with 1 mL of 2 % KI solution and 1 mL of 2 M HCl acid. The mixture was shaken vigorously until the appearance of yellow color and then added more with 0.5 mL of 0.01 % toluidine blue indicator, followed by 2 mL of 2 M NaOAc solution and mixed well. Absorbance was measured at 628 nm against reagent blank.

3.2.3 Analysis of pesticide

AOAC Official Method 2007.01 was applied to analyze pesticide residues in mango fruits by MeCN extraction; partitioning with magnesium sulfate ($MgSO_4$) and

quantifying by gas chromatography (GC) and high performance liquid chromatography (HPLC).

The analysis data of pesticide residue concentration were expressed in mg pesticide per 1000 g of mango or ppm and the degradation efficiency of pesticides was described by the percentage of reduction rate (Bai *et al*., 2010), which was calculated from the following equation:

$$\text{Reduction rate (\%)} = [(C_o - C_t) / C_o] \times 100$$

where C_o was the initial concentration of covered pesticide on mango without any treatment, and C_o-C_t was the removal concentration of pesticide with a give treatment.

3.2.3.1 Sample extraction and clean up

After NTP treatments, mango skins were immediately collected, chopped and crushed well by pestle and mortar. Pesticide residues in samples were analyzed according to the QuEChERS (quick, easy, cheap, effective, rugged, and safe) procedure (AOAC, 2007). Briefly, 5 g of well-chopped mango sample was weighed in a 15-mL centrifuge tube, then 5 mL of 1 % acetic acid (HOAc) in MeCN and 2.5 g anhydrous magnesium sulfate/sodium acetate ($MgSO_4$/NaOAc) (4/1, w/w) are added and the tube was closed. The mixture was shaken vigorously by a vortex for 1 min and centrifuged for 10 min at 5000 rpm. Then, 1 mL of MeCN extract (supernatant) was moved to the centrifuge tubes (Q-sep® QuEChERS dSPE Cat.#26219, Restek) containing 150 mg $MgSO_4$, 50 mg primary secondary amine (PSA), 50 mg C_{18} and 50 mg graphitized carbon black (GCB), mixed by a vortex for 30 s and centrifuged again for 10 min at 10000 rpm. This final extract was filtered through 0.45 μm membrane (Millipore) using a filtration syringe system, then transferred to auto sampler vials and stored at -18^{o}C until being analyzed by GC and HPLC.

3.2.3.2 GC analysis

Chlorpyrifos: An Agilent 6890A GC (Agilent Technologies, Palo Alto, CA, USA) coupled with an Agilent 7683B auto-sampler and a flame photometric detector (FPD) was employed to analysis chlorpyrifos residues in mango samples with and without NTP treatments. A RTX®-OPP2 column Restek (30 m, 0.32 mm ID, 32 μm, Cat.#11241) was used. The detector and injector temperature were set at 250^{o}C and

200°C, respectively. The injection volume was 1 µL in splitless mode. Helium (He) was used as the carrier gas and the make-up gas at 3.0 mL/min. Hydrogen (H_2) and air were used as the detector gases at 75.0 and 100.0 mL/min, respectively. Samples were analyzed following temperature program: the initial temperature was 80°C, hold for 1 min, increased to 280°C at 12°C/min, held for 10 min.

Cypermethrin: Cypermethrin residue concentration was analyzed by applying the Rtx®-5 column Restek (30 m, 0.25 mm ID, 0.25 µm df, Cat.#10223) and Shimadzu GC14B system equipped with an Agilent 7683B auto-sampler and an electron capture detector (ECD). The detector and injector temperature were set at 320°C and 250°C, respectively. The injection volume was 1 µL. Nitrogen (N_2) was used as the carrier gas at 1 mL/min. The column temperature program was as follows: the initial temperature was 150°C, hold for 1 min; increased to 250°C at 5°C/min, held for 8 min, and finally, increased to 300°C at 8°C/min, held for 2 min.

3.2.3.3 HPLC analysis

HPLC (Aglilent 1200, USA) system equipped with a diode array detector set at 286 nm and LiChrospher® RP-18 column (250 mm × 4 mm ID) was applied to analyze residue concentration of carbendazim. MeCN : DI water (1:1) was used as mobile phase with a flow rate of 0.9 mL min^{-1} and 10 µL volume of samples was injected into HPLC for each measurement.

3.2.4 Evaluation physicochemical characteristics of mango before and after treated with NTP conditions for decontamination covered pesticides

3.2.4.1 Physical quality indexes

Skin color, texture, moisture content, total soluble solid and total titratable acidity of mango (raw fresh material) and the fruits treated with NTP conditions (Table 3.1) for degradation of pesticide residues were evaluated. Three replications were done for each treatment.

Color measurement: The CIE (L*, a* and b*) of skin color of mango was determined at selected points on both sides of the fruit using a colorimeter (CR-410, Konica-Minolta, Japan) with ten measurements per fruit. The overall mango color

change after treatments was indicated by ΔE* which calculated according to the following equation:

$$\Delta E^* = \sqrt{(L_c^* - L_t^*)^2 + (a_c^* - a_t^*)^2 + (b_c^* - b_t^*)^2}$$

where L^*_c, a^*_c and b^*_c are the color values of control sample (mango fruits without any treatment), and L^*_t, a^*_t and b^*_t are the color values of fruits with treatments (Siddiq *et al*., 2013; Ma *et al*., 2015b).

Texture analysis: Mango samples were equilibrated to room temperature (28°C) and cut into uniform cubes (1.5 × 1.5 × 1.5 cm^3) before taking textural analysis. Texture of mango was evaluated by applying texture profile analysis (TPA) with a texture analyzer (TA-XTplus, Stable Micro Systems, UK) following to the method of Banjongsinsiri *et al*. (2004). A cylindrical probe (50 mm diameter) was chosen to perform TPA tests. Samples were compressed in two consecutive cycles to get 25 % deformation from the initial sample height of 1.5 cm, at a speed of 2 mm s^{-1}. The textural parameters determined were hardness, cohesiveness, springiness, gumminess and chewiness.

The first maximum force which is necessary to compress the mango cube sample is defined as the hardness and measured in newtons (N). The ratio of the area of work during the second compression divided by the area of work during the first compression is the cohesiveness. Springiness is the distance (mm) at which the sample was compressed by the peak force of second compression. The product of hardness and cohesive is the gumminess (N) while chewiness (N × mm) is calculated from gumminess and springiness. For each treatment, at least fifteen samples were performed.

Moisture and total soluble solids content measurement: The moisture content was determined according to AOAC (2000), and total soluble solids (TSS), expressed as °Brix, were measured with a refractometer (Atago, Tokyo, Japan) at 25 ± 1°C (Siddiq *et al*., 2013).

Total titratable acidity (TA) measurement: Titratable acidity (TA) was determined by titration with NaOH 0.1 N and two to three drops of 0.1 % (w/v)

phenolphthalein as an indicator (AOAC, 2000). The results were expressed as percentage of citric acid (%).

3.2.4.2 Chemical quality indexes

Chemical quality parameters of mango fruits including ascorbic acid, total carotenoid and total phenolic content before (fresh material) and after treated with NTP conditions (Table 3.1) for degradation of pesticide residues were determined. Three replications were done for each treatment.

Ascorbic acid content measurement: Ascorbic acid content in the mango flesh juice will be determined by titrating 20 mL of flesh sample juice with the 2, 6-dichlorophenolindophenol dye following AOAC (2000).

Total carotenoid content (TCC) measurement: Total carotenoids content (TCC) was determined following to method of Sellamuthu *et al*. (2013) with some slight modifications. Blended mango flesh (5 g) were mixed with acetone (10 mL) and shaken continuously for 3 h to extract carotenoids. Subsequently, the volume of extract was filtered and added with 10 mL petroleum ether, then the acetone was removed from the mixture by rinsing three times with distilled water. After that, the petroleum ether extract containing carotenoids was collected and passed through a funnel holding anhydrous sodium sulfate. The measurement was done by spectrophotometer (PerkinElmer Lambda 25, UV/Vis Spectrophotometer) at 453 nm and the blank sample was petroleum ether. TCC was expressed as mg/100g fresh weight (FW).

Total phenolic content (TPC) measurement: Blended 2.5 g mango flesh were added into 10 mL of ethanol-acetone solution (ratio 7:3) and kept in ultrasonic bath for 1 h to extract followed by the centrifugation process at 10,000 rpm (Hettich® Universal 320/320R Centrifuge, Tuttlingen, Germany) in 15 min. Supernatant was collected and analyzed of total phenolic content. Total phenolic content were determined following Siddiq *et al*. (2013). Taking 0.5 mL of standard or extracted sample and 0.5 mL of Foline-Ciocalteu reagent (diluted 10 times with distilled water) into small test tubes, the mixture was shaken well for 15 s. After 3 min, adding 1 mL of saturated sodium carbonate (about 75 g/100 mL) and 1 mL of distilled water into these tubes. The mixture was incubated in the dark area for 2 h before its absorption was

measured at 725 nm against de-ionized water by spectrophotometer (PerkinElmer Lambda 25, UV/Vis Spectrophotometer). The data were expressed in mg gallic acid equivalents (GAE)/ 100g fresh weight (FW).

3.2.5 Evaluation of sensory properties of mango fruit

The sensory properties including color, aroma, flavor, texture and overall-liking of mango samples treated with the best conditions of NTP applied to decontaminate pesticide residues were evaluated and compared with raw fresh mango fruits and the fruits treated with conventional hot water method using 9-point hedonic scale of 50 panelists.

The hot water treatment was conducted by submersing the mangos in a hot water bath (Memmert GmbH, Schwabach, Germany) at 55^{o}C for 5 min, then the fruits were immediately cooled with cold water at 10^{o}C for 15 min and air dried at 28-30^{o}C for 30 min (Sripong *et al*., 2015).

3.3 Effects of NTP on the inactivation of *C. gloeosporioides* causing anthracnose disease of mango fruit

The 7-day fungus culture grown on PDA was used to assess the effects of NTP on this pathogen causing mango anthracnose disease. Details about sample preparations for NTP treatments were described in the following sections.

3.3.1 NTP treatment

To evaluate the effect of NTP on *C. gloeosporioides* inactivation, the same NTP system design from Kim *et al*. (2013) for degradation pesticide residues covered on mango was applied except for the reservoir tank containing distilled water which was removed.

The plasma-treated Ar gas and micro-bubble water vapor after passing the GA discharge system (8 kV and 0.6 A) was used directly to treat samples (depicted in Figure 3.3) including petri plates containing mycelial plug of *C. gloeosporioides*, test tubes holding the fungal spore suspension, and inoculated mango fruits with this anthracnose mold.

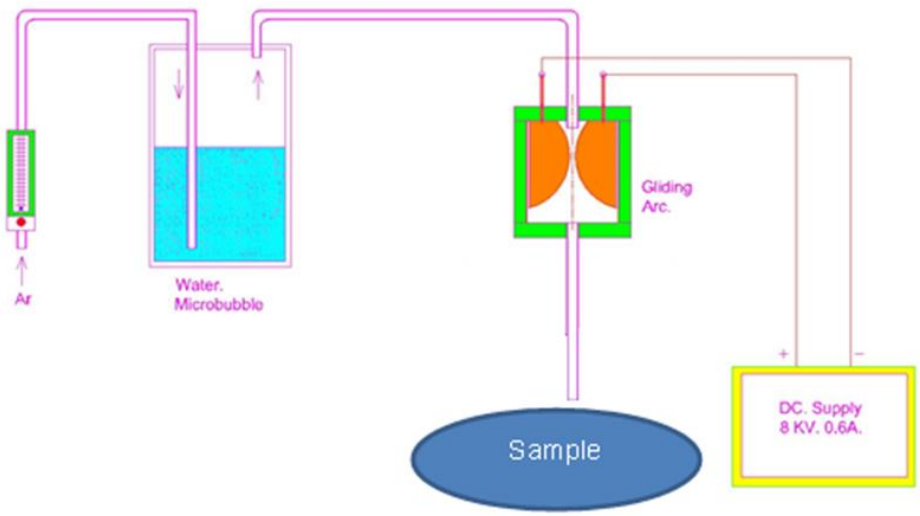

Figure 3.3 Schematic of NTP system for inactivation of *C. gloeosporioides*

3.3.2 Assessment of NTP effect on *C. gloeosporioides* mycelial growth

Following to the method of Thinh and Kunasakdakul (2013) with some slight modifications, the 0.5-cm diameter of fungal plug cut from a seven-day culture of *C. gloeosporioides* was placed at the center of the PDA medium amended with streptomycin sulfate (approximately 100 mg/L) to suppress bacterial growth (Ekefan *et al*., 2000) in 8.5-cm diameter petri dish. After that, the petri dish with the mycelial plug was exposed to NTP (Figure 3.3) generated by applying different flow rate of Ar gas ranged from 1-5 L/min through GA discharge for duration time from 1-7 min with a distance of 0.5 cm from the tip of gliding arc discharge. NTP treatment conditions for inhibition mold mycelial growth on petri plates coded and showed in Table 3.2. Four replications were done for each treatment. The PDA plates were incubated at room temperature (28-33°C) and the average diameter of the fungal growth was measured and recorded every day using a digital Vernier caliper (Mitutoyo, Japan) until the fungus reached the edge of the plate.

3.3.3 Evaluation of NTP effect on *C. gloeosporioides* spores in suspension

A spore suspension was prepared by scraping spores off the seven-day-old cultures of *C. gloeosporioides* grown on PDA agar with the aid of a glass rod and suspending them in sterile distilled water (Thinh and Kunasakdakul, 2013). The mycelium and spore mixture was filtered through cheesecloth, diluted 3 times with

sterile water and transferred into sterilized test tubes (each containing 2 mL of spore suspension). After that, the glass tube with 2 mL of *C. gloeosporioides* spores suspension was exposed to NTP (Figure 3.3) generated by applying different flow rate of Ar gas ranged from 1 to 5 L/min through GA discharge for duration time from 1 to 7 min with a 0.5-cm distance from the tip of gliding arc discharge to the surface of spore suspension solution. NTP treatment conditions for reduction of mold spore in water were similar to those for inhibition the mycelial growth on petri plates which were coded and showed in Table 3.2. Three replications were done for each treatment. The number of *C. gloeosporioides* spores before and after NTP treatments was counted by using a hemocytometer (Boeco, Germany) and reported as log spore/mL.

Table 3.2 Conditions and codes of different NTP conditions to treat mycelium on PDA agar and spore suspension of *C. gloeosporioides*

Test ID	Flow rate of Ar gas (L/min)	Plasma treatment time (min)
Control	Petri dish with fungal plug or glass tube containing spore suspension without any plasma treatment	
1L_1min	1	1
1L_3min	1	3
1L_5min	1	5
1L_7min	1	7
3L_1min	3	1
3L_3min	3	3
3L_5min	3	5
3L_7min	3	7
5L_1min	5	1
5L_3min	5	3
5L_5min	5	5
5L_7min	5	7

3.3.4 Evaluation of NTP effect on anthracnose disease severity

Following method described by Jitareerat *et al*. (2007) and Zheng *et al*. (2013) with slight modifications, mango fruits were firstly washed with sterile distilled water

to remove dust and then the fruits were surface sterilized by wiping with sterile tissue paper soaked with 70 % ethanol. After drying, each mango fruit was wounded using a sterile needle and artificially inoculated with a 0.5-cm mycelial plug cut from a seven-day culture of *C. gloeosporioides*. The inoculated mangoes were placed into plastic boxes (1 mango per box) with high relative humidity (95 % of RH). The lids were put on and the mangoes were incubated at room temperature (28-33^{o}C).

To evaluate the impact of NTP on disease severity caused by *C. gloeosporioides*, the inoculated mango were exposed to the selected NTP conditions showing the good results to inhibit the growth of mold mycelial as well as high impact on spore suspension before placing into plastic boxes and incubating at the same condition with the control which was not received any NTP treatment.

The severity of disease in the mango fruit was assesses every day from third day to twelve day of incubation by the extent of the total decayed area on each fruit surface using a 5-point scale, where 0=no disease symptoms, 1=1-10 % of disease symptoms, 2=10-20 % of disease symptoms, 3=20-30 % of disease symptoms, 4=30-40 % of disease symptoms, 5=>40 % of disease symptoms on the affected fruit surface (Sripong *et al*., 2015). Percentage of disease symptoms was calculated from the following equation:

$$\text{Disease symptom percentage (\%)} = (A_1/A_o) \times 100$$

where A_o was the surface area of mango fruit, and A_1 was the lesion area of the fruit caused by *C. gloeosporioides*. Each treatment contained three replicates, and each replication had three fruits.

Measurement of $OH^{\bullet}$ radicals emission signal and concentration of hydrogen peroxide (H_2O_2) were also measured. Emission of $OH^{\bullet}$ radicals and concentration of hydrogen peroxide (H_2O_2) generated according to NTP conditions (Table 3.2) to inhibit *C. gloeosporioides* were also evaluated with the same methods of measuring those for NTP conditions to degrade pesticide residues covered on mangoes.

3.4 Statistical analysis

Data analysis was performed using SPSS statistical package 16.0 (SPSS Inc., USA) according to completely randomized design (CRD). The influence of NTP

treatment conditions including flow rate of gas and treatment time on degradation of pesticide residues and inhibition of *C. gloeosporioides* were evaluated. Mango sensory properties and fruit quality attributes including color, texture, moisture content, TSS, TA, ascorbic acid content, TCC and TPC of the control and plasma treated samples were tested for significance ($p \leq 0.05$) by one-way analysis of variance (ANOVA) and Duncan's post hoc test.