CHAPTER 3

Materials and Methods

1. Incidence and distribution of viral diseases of cucurbits in the Royal Project's areas

1.1 Collection of the diseased samples

A survey of major viral diseases of cucurbits in the Royal Project's areas which are in the highland of northern Thailand was conducted in various locations and different seasons. The diseased samples with various symptoms were collected from zucchini, Japanese pumpkin and Japanese cucumber from the main cucurbit growing areas during the surveys conducted in 2008-2009. The zucchini samples were collected from three Royal Project Development Centers (RPDCs); Mae Hae and Mae Tha Nuea in Chiang Mai Province. The pumpkin samples were collected from five RPDCs; Mon Ngo, Wat Chan, Mok Cham and Kae Noi in Chaing Mai province and Mae La Noi in Mae Hong Son province. The cucumber samples were collected from four RPDCs; Mae Tha Nuea, Huai Luk, Mae Sa Mai and Mae Phae in Chaing Mai province (Figure 3.1). Each field was inspected during the early-developmental stage through the end of the crop. Samples with characteristic virus-like symptoms such as systemic mosaic, vein clearing, blistering, severe yellowing symptoms, and deformation were collected. The sample consisted of two to three leaves and fruits from individual plants showing distinct symptoms of virus infection on the leaves, as well as at the level of the overall appearance of the plant. The samples were photographed to record the symptoms and then put in labeled plastic bags. The sample bags were kept in an ice box during transportation to the laboratory. The samples were stored in a refrigerator at 4 ^{o}C for 1-2 days before the serological tests were performed.

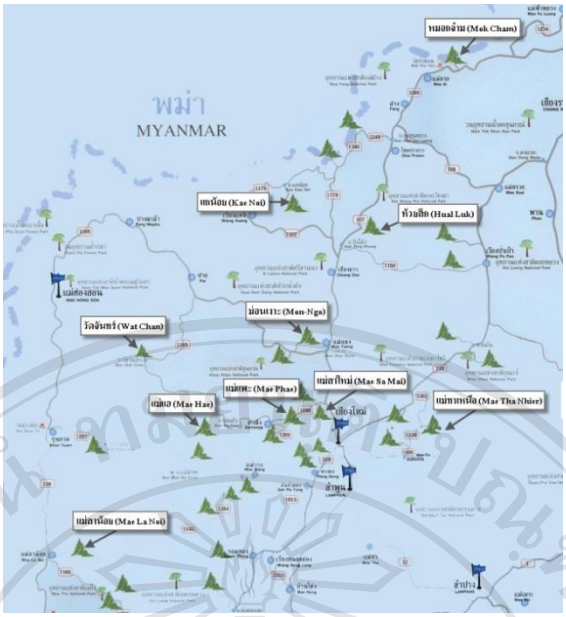

Figure 3.1 Map of a survey of major viral diseases of cucurbits in the Royal Project's areas (Modified from www. thairoyalprojecttour.com).

1.2 Detection of viruses by serological test

Identification of the viral pathogen from the infected samples was carried out using a serological method, the double antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA). Polyclonal antibody ELISA kits from Bioreba (Switzerland) were used for detecting cucumber mosaic virus (CMV), zucchini yellow mosaic (ZYMV), squash mosaic virus (SqMV), papaya ringspot virus type-W (PRSV-W), watermelon mosaic virus-2 (WMV-2), and Tospovirus. DAS-ELISA method was used to detect viruses following the protocol described by the company. Duplicate wells were used for each sample, healthy and diseased wells were also included on all ELISA plates (certified Nunc-Immuno Plates MaxiSorp F96). The ELISA plate wells were coated with 100 µl IgG (1 in 1,000 dilutions) according to manufacturer's specification in coating buffer and the plates were incubated at 30^{o}C for 4 h. The plates were washed three times with PBST washing buffer for three mins between each wash. The diseased samples were extracted with 0.02 M PBST (1:10 w/v), together with 0.05% Tween20, and 2% polyvinyl pyrrolidone MW40000. Using a pipette 100µl of the diseased sample sap was pipetted into each well and incubated overnight at 4 ^{o}C. Then the plates were removed and washed again as described. 100 µl of alkaline phosphatase IgGs at 1:1000

dilutions in conjugate buffer was pipetted into the wells and the plates were incubated at 30ºC for 5 h. The plates were washed as described before and 100 µl of 1 mg/ml p-nitrophenyl phosphate in substrate buffer were pipetted into the wells. The plates were incubated at room temperature for 60 min to obtain a clear reaction. The reaction was calorimetrically detected at A405 nm using an ELISA reader (Sunrise basic TECAN, Austria).

2. Distribution of insect vector and alternate host in the Royal Project's areas

2.1 Survey of insect vectors in main areas of cultivation

A survey of insect vectors of major viral diseases in cucurbits was conducted in 2008 and 2009 at various locations and in different seasons. Insect vectors found included aphids, thrips, whitefly, and cucurbit beetle. The vectors were collected from zucchini, Japanese pumpkin and Japanese cucumber fields from 9 RPDCs in two provinces; Mae Hae, Mae Tha Nuea, Huai Luk, Mae Sa Mai, Mae Phae, Mon Ngo, Wat Chan, Mok Cham and Kae Noi (Chiang Mai Province) and Mae La Noi (Mae Hong Son province).

2.2 Collection of alternate hosts of the viral diseases

Samples of the no cultivated plants showing virus-liked symptoms were collected in 2008 and 2009 in the areas where cucurbits were grown. The samples of alternate hosts included wild cucurbits and other different weed species and were collected from the rims and planting areas. The samples were photographed to record the symptoms before putting samples into labeled plastic bags. The samples were identified to species level and inspected for the presence of virus-like symptoms. The samples were brought to the laboratory for serological testing using the previously described procedures. The polyclonal antibodies ELISA kits were used to detect CMV, PRSV-W, WMV-2, ZYMV, SqMV, potato virus Y (PVY), and Tospovirus (Bioreba, Switzerland), CGMMV, melon necrotic spot virus (MNSV), watermelon silver mosaic virus (WSMoV), tobacco mosaic virus (TMV), tomato mosaic virus (ToMV), tomato spotted wilt virus (TSWV), pepper mild mottle mosaic virus (PMMoV), pepper mottle virus (PeMV), passion fruit woodiness virus (PWV), east

asian passiflora virus (EAPV), Geminivirus and Potyvirus (Agdia Inc., Elkhart IN., USA).

3. Studying the correlation between the aphid vector and the viral pathogen ZYMV

3.1 Collection of insect vector (aphids)

The aphids found feeding on cucurbitaceous crops with typical symptoms of ZYMV disease were collected and verified by ELISA kit (Bioreba AG, Switzerland). The aphids that carried ZYMV were transferred to healthy zucchini plants grown in an insect proof greenhouse. They were left to feed on the plants for an access period of 20-60 min. When the ZYMV infected zucchini showed symptoms, they were sampled for testing by ELISA. Numbers of aphids used for transmit the virus were 1, 5, 10, 15, 20, 25 and 30 aphids per plant. The objective was determined whether there was a correlation between number of aphid vector and expression of ZYMV disease symptoms.

3.2 Detection of viruses by serological tests

The aphids carried ZYMV collected from the infected zucchini plants were brought to the laboratory to check the virus. DAS-ELISA method is use to detect ZYMV (Bioreba AG, Switzerland). The serological test was carried out using the procedures previously described.

4. Detection of seed transmission or virus in cucurbitaceous crops (zucchini, Japanese pumpkin and Japanese cucumber)

4.1 Seed materials and experimental conditions

Commercial seeds of three cucurbitaceous crops; zucchini (*Cucurbita pepo*) cultivar SENATOR (hybrid squash, lot No.978883); Japanese pumpkin (*C. moschata*) cultivar DELICA (hybrid squash, lot No.08021); and Japanese cucumber (*Cucumis sativas*) cultivar PRETTY SWALLOW 279 (cucumber F1 hybrid, lot No. EA25061). The sample and subsample size to be tested followed ISTA (2010). Five seed groups were separated. Group 1, consisting of 400 whole seeds; group 2, the endosperm of 400

seeds; and group 3, the seed coat of 400 seeds, were soaked in 1X PBS buffer (0.02 M PBST (1:10 w/v) for a minimum of 1 h at 4°C. Solids settled to the bottom and the light-colored upper layer was used for viral detection. Two hundred seeds of Group 4 and 5were sown in plastic trays filled with a growing medium and placed in an insect proof greenhouse. The cotyledons of group 4 were collected for testing. The leaves of group 5 were randomly collected to be examined after the seedlings reached the trifoliate stage, 10 d after planting (DAP). Single cotyledons/leaves were individually ground in PBS buffer and centrifuged (10,000g) for 5 min. The supernatants of ground cotyledon/leaf tissues were used for virus detection.

4.2 Detection of seed transmission of viruses

DAS-ELISA method was used to detect CMV, ZYMV, SqMV (Bioreba AG, Switzerland) and CGMMV (Agdia Inc., Elkhart IN, USA) following the protocol as described by the respective companies. Sap from the five groups of seed was used for the DAS ELISA as previously described.

5. Examination of seed transmission of CGMMV and its detection at various growth stages of cucumber

5.1 Seed materials and experimental conditions

The commercial seeds of Japanese cucumber (*C. sativas*) cultivar PRETTY SWALLOW 279 (cucumber F1 hybrid, lot No. EA25061) was used throughout this study. The sample and subsample size to be tested followed ISTA (2010). Five seed groups were separated and tested for CGMMV using DAS-ELISA as previously described.

5.2 Collection of infected cucumber samples

Eight-day-old seedlings of Japanese cucumber were cultivated at three Royal Project Development Centers; Mae Tha Nuea that cultivated in field, Huai Luk that cultivated under greenhouse (the main cucumber growing areas) and Thung Roeng (not previously planted with cucumber) throughout May to July 2011. Leaf samples were taken from at least 100 plants at different growth stages, seedling, flowering and fruiting

stages (1 month after fruit set), and symptoms were visually categorized as mosaic, mottle, chlorosis, leaf deformation and/or stunting.

5.3 Fruit production

The effect of CGMMV and stage of infection in cucumber planted at the three different locations was evaluated. Fruits were collected every 1 or 2 d from 100 plants at 12-14 d after fruit set. Marketable fruit sizes were classified into three standard grades; grade 1 was 15-20 x 3.0-3.5 cm, grade 2 was 15-20 x 2.5-3.5 cm. and under grade was >15 cm. Others were unmarketable or rejected grade.

6. A study of correlation between viral combinations and symptoms on zucchini

6.1 Isolation and purification of plant virus pathogen

Pure isolates of viruses were obtained from naturally infected cucurbits at 10 RPDCs. Inoculations of the pathogenic viruses to *Chenopodium amaranticolor* were made by mechanical transmission for 3 consecutive times. When the inoculated leaves showed localized symptoms, the virus was identified by ELISA kit (Bioreba AG, Switzerland and Agdia Inc., Elkhart IN, USA). The introduced viruses were maintained in the zucchini plants under insect proof greenhouse condition.

6.2 Detection of viral combination on zucchini

Leaf samples from virus infected zucchini were ground in 0.02 M potassium phosphate buffer (1:10) at pH 7.0. The extracts plus carborudum were used to inoculate 8-day-old zucchini seedlings by smearing the extract on the cotyledons. Single and mixed infection of virus, e.g. ZYMV, CMV, PRSV-W, WMV-2 and CGMMV, were evaluated. When the viral disease symptoms appeared, they were photographed to record the symptoms before being assayed by DAS-ELISA.