AGF/CRO/11/007/REG	DATE: 25 OCTOBER 2015

JAIF PROJECT PROGRESS REPORT

PROJECT TITLE:	**Taxonomic capacity building to support market access for agricultural trade in the ASEAN region**	
PROJECT PROGRESS REPORT:	☐ 1st XXX ☐ 3rd ☐ 4th ☐ 5th	
PROJECT START AND END DATES	From: MAY 2015	To: APRIL 2017
PERIOD COVERED BY THIS REPORT:	From: 02 NOVEMBER 2015	To: 30 APRIL 2016
IMPLEMENTING AGENCY:	ASEAN Plant Health Cooperation Network (APHCN) - ASEANET	
CONTACT PERSONS:	Names: DR LUM KENG YEANG (Chairperson & Project Manager) & DR SOETIKNO S. SASTROUTOMO (Technical Secretary) Tel: +60-3-8943-2921 Fax: +60-3-8942-6490 E-mail: ky.lum@cabi.org AND s.soetikno@cabi.org	

OVERVIEW:

Briefly describe: (i) the objective of the project; (ii) progress in project implementation to date; (iii) any particular issues faced and/or results achieved during this reporting period.

(i) **Overall objective:**
The project will develop and strengthen capacities in taxonomic knowledge to identify and manage quarantine risks associated with agricultural commodities and to accurately diagnose pests and diseases among the ASEAN Member States (AMS).

Intermediate objective: To increase taxonomic capacity of scientists/officers from AMS in 3 groups of insect pests and diseases, i.e. in plant viruses, aphids and leaf miners of agricultural importance.

(ii) **Progress till April 2016:** Two major activities have been carried out in the period from November 2015 to April 2016; Activity 1.1. A Training Workshop on Diagnostics of Leafminers of Agricultural Importance held at the Museum Zoologicum Bogoriense (MZB), LIPI, Cibinong, Indonesia from 29 February to 11 March 2016 and Activity 1.2. Attachment Program on Plant Viruses held in the Graduate School of Agriculture, Tokyo University of Agriculture (NODAI), Tokyo, Japan from 26 October to 25 December 2015. In addition the website for this project has been updated with uploading of the 1st Progress Report of the Project, training materials, and reference materials. Copies of the project brief were distributed to the JAIF Leafminers workshop participants and also to participants of 3 (three) AANZ-FTA workshops, i.e. on Immature Lepidoptera, Whiteflies and Diagnosticians Forum.

(iii) **Results:**
1. Report of the Attachment Program in Japan
2. Report of the Training Workshop on Diagnostics of Leafminers of Agricultural Importance
3. Project Website (http://aseanet.org/JAIF1.asp)

PART A: PROGRESS & RESULTS

A. PROGRESS & ACHIEVEMENTS:

Describe progress in implementation during this reporting period, including key outputs/outcomes, based on the approved project document.

Attachment Program in Tokyo University of Agriculture, Japan

The attachment program was held at the Laboratory of Tropical Plant Protection, Tokyo University of Agriculture (Tokyo NODAI), Japan from October 26 – December 25, 2015 with the objective to strengthen understanding of the concepts and practice with plant viruses, a diagnosis of infected plants, and to learn the latest technology and management of diseases attributed to plant viruses.

The attachment were participated by 3 (three) plant health/biosecurity officers, one each from Indonesia, Malaysia and Vietnam, i.e.

1. Ms. Nur Fitriawati, BBUSKP, Plant Quarantine and Biosafety Centre, Indonesian Agriculture Quarantine Agency (IAQA), Jakarta, Indonesia
2. Ms. Norhayati binti Madiha, Agriculture Officer Plant Biosecurity Division Department of Agriculture Jalan Gallagher, 50632 Kuala Lumpur Malaysia
3. Mr. Tran Van Chien, Post Entry Quarantine Centre I, Plant Protection Department, Ministry of Agriculture & Rural Development, Hanoi, Vietnam

During the attachment program they were supervised by Prof. Keiko Natsuaki from Laboratory of Tropical Plant Protection, Tokyo University of Agriculture, Japan. The selection of participants was based on the individual performance and evaluation results as well as observation and evaluation by all resource persons during the "Training Workshop on the Diagnostic of Plant Viruses" that was organised at UPLB, Philippines from 17-28 August 2015. The program of the 2 months attachment comprised four components, i.e.:

a). Series of lectures given by resource persons and invited speakers,

b). Individual experiments and laboratory work supervised by Prof. Natsuaki and Dr. M. Pinili,

c). Participation in the International Congress of International Society for South East Asian Agricultural Sciences (ISSAS) organized by Tokyo NODAI from 7-9 November 2015, and

d). Participation in the seminar and defence of PhD students of Tokyo NODAI.

The full reports prepared by Prof. Natsuaki and the 3 participants are given in the **Attachments 1-4.**

Training Workshop on Diagnostics of Leafminers of Agricultural Importance

This "Training Workshop on the Diagnostics of Leafminers of Agricultural Importance" was coordinated by the Museum Zoologicum Bogoriense, Centre for Research Development in Biology, LIPI, Cibinong, Bogor, Indonesia from 29 February to 11 March 2016 and was participated by 20 (twenty) entomologists from Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Singapore, Thailand and Vietnam. The majority of them are attached to Plant Quarantine/Biosecurity/Plant Health and Plant Protection Centres of their respective countries under their Department of Agriculture.

The ultimate goal of the training workshop was to develop capacity among entomologists across the ASEAN region in addressing leafminer pests in each country that may pose potential threats (emerging or invasive) to their economic crops intended for export or as planting materials. The training workshop provided the participants with basic knowledge on the ecology and taxonomy of leafminers of agriculture importance with focus on improvement of skills in the identification of leaf miners and their parasitoids using morphological characters and molecular technique. The main resource persons of the workshop were from Nara Women University, Nara, Japan (Prof. Hiroaki Sato); School of Applied Systems Biology, Centre for Agri-Bioscience, La Trobe University, VIC 3083, Australia (Assoc. Prof. M.

Malipatil), and from the Museum Zoologicum Bogoriense Bogor (Dr. Hari Sutrisno, Prof. Rosichon Ubaidillah and Dr. Awit Suwito and his team). The full report of the Training Workshop is given as **Attachment 5.**

Project Brief

Twenty five (25) copies each of the Project Briefs has been distributed to participants at the following events:

1. JAIF Training Workshop on Diagnostics of Leafminers of Agricultural Importance (LIPI, Cibinong, Indonesia, March 2016)
2. AANZFTA - Workshop on Diagnostics of Immature stages of Pest Lepidoptera (Bekasi, Indonesia, November 2015)
3. AANZFTA – ASEAN Diagnosticians Forum (Putrajaya, Malaysia, December 2015)
4. AANZFTA – Weed Identification and Risk Assessment Workshop (Vientiane, Laos, February 2016)

JAIF Project Website – Component 2 of the Project (see Attachment 6)

Objectives of this component include information sharing, information dissemination and mainstreaming and institutionalisation of information through networking. The information gained through networking and exchange of information becomes embedded and enters the common knowledge domain of respective institutions and NPPOs of countries concerned.

Mainstreaming and institutionalisation of information is achieved through the various activities in the project; training and capacity building workshops, attachment programs with experts, engagement through the project website, online tools and services and other project activities.

The project website provides a platform to host tools and services e.g. expert register databases, online diagnostic tools, pests and diseases information etc.

To raise awareness and disseminate information, the use of offline and online media will be used. Marketing and promotional materials and collaterals may come in the form of flyers, posters, brochures, online web feeds, or e–newsletters which will be produced and distributed.

B. TIMEFRAME AND BUDGETING

Explain whether the project is on-track with regard to: (i) the budget; and (ii) the original timeframe. If either the expenditures and/or timeframe are off-track, please explain and describe the corrective actions being taken.

Project implementation is on track with 3 major activities, i.e. Attachment Program on Diagnostics of Plant Viruses in Japan, Training Workshop on Diagnostics of Leafminers, and updating of the JAIF project website. One activity, i.e. Attachment Program on Leafminers is in progress. Three participants for this program would be selected by end of April and travel arrangements would be made in May. They are scheduled to depart for Nara, Japan in August for 2 months.

Budget expenditures are also on track and without any over-expenditure in all activities that have been carried out so far **(Attachment 7)**.

C. OTHER IMPLEMENTATION ISSUES

Describe any significant changes to the project design, context or partners during the reporting period, or any other issues faced, and actions that are being taken in response, if appropriate.

No significant changes to the project design, context or partners in this reporting period.

D. OTHER COMMENTS:

Please provide any other relevant information or observations on the project, e.g. on lessons learned, particular challenges or issues that may arise in the next reporting period, changes to the logframe, etc.

None.

Provide a list of key documents (e.g. mission reports, training materials, workshop reports, etc.) produced during this reporting period. Copies of the final versions of these documents should be attached to this report.

1. Report of the Attachment Program on Diagnostics of Plant Viruses (Attachments 1-4)
2. Report of the Training Workshop on Diagnostics of Leafminers of Agricultural Importance (Attachment 5)
3. Report of the activities of Component 2 – Project Website (Attachment 6)
4. Financial Summary until 30th April 2016 (Attachment 7).

PART E: FINANCIAL OVERVIEW (SEE ATTACHMENT 7)

	JAIF*	In kind / Other**	Total
a) Total project budget (US$)		-	
b) Total amount received to date (US$)			
c) Total expenditure during the reporting period*			
d) Total expenditure to date (US$)			
e) Unspent funds a) – d) (US$)		-	

*Based on current rate US$1.00 = RM3.37364

[a)] One of the regional expert (Dr. M. Malipatil) was sponsored (airticket, consultation fee and DSA) by AANZ-FTA Program, Australia.

Attachment 1

Two Months Attachment Program on Diagnostics of Plant Viruses

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan

October 26 – December 25, 2015

Organized by:

Tokyo University of Agriculture (Tokyo NODAI), Japan

In Collaboration with:

ASEAN Network on Taxonomy

2016

Two Months Attachment Program

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan
October 26 – December 25, 2015

Attachment Report

by
Prof. Dr. Keiko T. NATSUAKI
Tokyo University of Agriculture

Background

Plant viruses cause various diseases and give strong impact on agricultural production, conservation of plant resources and international plant quarantine. Symptoms caused by plant viruses are various and virus diseases are known to responsible for crop yield loss both in quantity and quality. We, however, have almost no effective treatments against plant viruses and this is different situation from fungal and bacterial diseases against which we have effective fungicides and antibiotics. In this regards, proper management and application of available control measures upon early diagnosis are most significant.

In advance, "Training Workshop on Diagnostics of Plant Viruses" funded by Japan-ASEAN Integration Fund (JAIF) through ASEAN Plant Health Cooperation Network of the ASEANET (APHCN-ASEANET) have been successfully carried out by Dr. Marita S. Pinili, Training coordinator, UPLB, and Dr. Soetikno S. Sastroutomo, Secretary, APHCN-ASEANET at University of the Philippines at Los Banos for 2 weeks in August 2015 in which I joined as one of the resource speakers from Aug 17 to 24. Then I was invited to organize the "Follow-up Attachment Program" for two months at my laboratory for three selected participants from the previous mentioned workshop.

Objectives

At the "Training Workshop on Diagnostics of Plant Viruses" in August 2015, all the participants learnt basic technology needed for diagnostics of plant viruses. Plant viruses are diverse and cause disease in many important crops. As it is almost impossible to eliminate viruses, early and accurate diagnosis is most requested in agricultural sectors and plant quarantine systems. In this regards, more advanced technology for diagnostics of plant viruses is necessary. Objectives of this "Follow-up Attachment Program" for selected participants are to help them to master advanced technology and give them confidence in their technology to disseminate it to their colleagues.

Contents of the program

1) Lectures were given for participants;
   - General guidance of the program
   - History a and education policy of Tokyo NODAI
   - Molecular detection of plant viruses
   - Nematology by Dr M.S.Pinili
   - Use of database and phylogenic analysis by Dr. N.Furuya

2) Experimental courses or laboratory works were given by me, Dr. Pinili, Dr. T.Natsuaki and with the help of several graduate students of Tokyo NODAI.
   - Artificial inoculation including preparation of inoculation buffer and index plants.
   - Aphid inoculation of banana viruses.
   - RT-PCR detection of the genus Potyvirus and *Cucumber mosaic virus* including preparation of necessary solutions.
   - PCR detection of DNA virus such as *Banana bunchy top virus including preparation of necessary solutions.*
   - Electron microscopic observation technology including preparation of samples and PTA staining solution.
   - ELISA for Potyvirus using several samples such as bamboo, passion fruits, papaya and others. Preparation and results analysis were also included.
   - Gel-electrophoresis for protein analysis.
   - FTA sheets sample storage
   - Detection of ds RNA for unknown viruses at Utsunomiya University on December 3-5.

As for PCRs and ELISA, experiments were conducted repeatedly until participants can carry out the whole experiments by themselves.

3) Participation to an international congress

Three participants joined International Congress of International Society for South East Asian Agricultural Sciences (ISSAS) which was held at Tokyo NODAI from November 7 to 9 including field excursion. Participants learnt general agricultural sciences in SE Asia by key note and other lectures, and poster sessions. They could have good communications with scientists from various countries.

4) Participation to a defense for PhD degree on plant virology

Three participants joined the final 40 minutes presentation of a PhD candidate on her *Rice yellow mottle virus* work on December 17.

5) Visit to Plant Quarantine office and research institute

Three participants visited above mentioned office and institute in Yokohama and learnt by a short lecture and guide on their activities on December 11.

6) Final presentation using power points by each of the participants.

On December 21, three participants gave oral presentation on plant quarantine in each of their country and also the outcome of this program for 20 min each.

7) Participation to several events such as scientific presentation at Tokyo NODAI university festival on November 1, the farewell party for Dr. Pinili, and the farewell party for themselves on December 21. Communication with Japanese students and also international students from Afghanistan, Taiwan, Uganda and others were made friendly and students were invited to work together at the time of experiments.

Outcomes

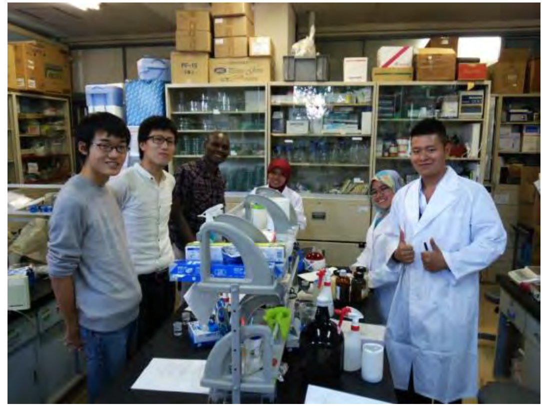
I highly evaluated the excellent ability and sincere attitude of three participants, Mr. Tran Van Chien, Ms. Norhayati Binti Madiha, and Ms. Nur Fitriawati Msi, which they showed during the program. I can proudly report here that they performed as they were expected wonderfully.

1. By a few lectures and talks during the experiments, the participants could learn more advanced knowledge and information on plant viruses which is necessary to master plant virus diagnostics.
2. By experiment course, the participants mastered advanced plant virus diagnostic techniques. They can conduct inoculation, aphid transmission, ELISA, RT-PCR, ds-RNA detection, gel electrophoresis and electron microscopy by themselves only if they have necessary facilities and chemicals.
3. The participants improved their ability and deepen their knowledge on plant viruses by lectures and discussion with resource speakers.
4. The participants understood more about plant quarantine system in Japan by the visit to Yokohama Plant Quarantine in Japan as well as systems in Vietnam, Malaysia and Indonesia by exchange of information among the participants.
5. The participants learnt laboratory management of Tokyo NODAI and also Japanese culture. They are expected to be the bridge between ASEAN countries and Japan.

Problems and suggestions

1. Three participants were selected in this program and they worked really hard. I hope in next program, however, not only three but at least four or more persons will be selected. The reason one is that odd numbers of participants are not possible to be grouped evenly at the time of experiments. The reason two and the most important reason is that this program is very significant to all plant quarantine officers in ASEAN countries and thus I hope more people can join this program.
2. I would like to suggest to organize training programs such as a training program for diagnostics of Begomoviruses and a training program for diagnostics of phytoplasma. Begomovirus is a group of emerging and whitefly vectored plant viruses with DNA genome. This group is devastating in many crops and thus diagnostics of this group is very important. As for phytoplasma, disease occurrence is not common but we need special technology in diagnostics which is different from diagnostic technology of viruses.
3. To maintain the network and communication among participants, I hope we can exchange plant virus or even personal information from time to time by email.

Acknowledgement
I thank Japan-ASEAN Integration Fund (JAIF) for their strong and generous financial support to this program in Tokyo. It was a wonderful experience to work with Dr Soetikno S. Sastroutomo (APHCN-ASEANET) and I thank him a lot for bringing me the opportunities to carry out this program. Many thanks go to Dr. M.S.Pinili for her excellent contribution as a resource person in giving the vivid and fruitful experiment course to the participants. I also thank Dr. T.Natsuaki, Utsunomiya University for accommodation of 3 participants for dsRNA experiments and Dr. N. Furuya for her attractive lecture on phylogenic analysis. In every program including lectures, laboratory sessions, visit to institutions, parties, my students and I shared unforgettable experience with Dr Pinili and three trainees. I would like to also thank Ms. Yoko Otsuka, NODAI Research Institute, for her kind and careful work for all accounting procedures and Mr. Chung Ilsung for his constant support in the laboratory during this program.



The first day of the training with Japanese and Ugandan students at Tokyo NODAI (right)
Observation of virus diseases in the campus with a Tokyo NODAI student (left)

Experiment training (left) and after the seminar (right) with Dr. M.S Pinili.

With Dr. N.Furuya after her lecture and workshop.

With Dr. T.Natsuaki and students of Utsunomiya University and Tokyo NODAI at Utsunomiya University, Utsunomiya.

A group photo at the visit to Yokohama Plant Quarantine Office, MEXT, Japan.

At the SAYONARA party with the students and staffs of the laboratory of tropical plant protection.

Attachment 2

Report of Attachment Program
Advanced Diagnostics of Plant Viruses

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan
October 26 – December 25, 2015

By

Nur Fitriawati
(Indonesia)

Organized by:

Tokyo University of Agriculture
(Tokyo NODAI), Japan
In Collaboration with:

ASEAN Network on Taxonomy

2016

ATTACHMENT PROGRAM:

Advanced Diagnostic on Plant Viruses

Taxonomic capacity building to Support Market Access for Agricultural Trade in the ASEAN Region

Tokyo University of Agriculture, Japan

Duration:
Two Months (26th October-25th December 2015)

Participant Name and Positions:
Nur Fitriawati
Quarantine Officer

Institutional Address and Country:
Balai Besar Uji Standar Karantina Pertanian
(Center of Diagnostic Standard of Agricultural Quarantine)
Jalan Pemuda No. 64, Kav. 16-17, Rawamangun, Jakarta Timur
Indonesia

INTRODUCTION

Globalization has provided a dynamic platform to sustain high and durable rates of economic growth and social development for ASEAN country. One of the purposes of ASEAN Free Trade Area (AFTA) is to increase ASEAN's competitive edge as a production base in the world market through the elimination, within ASEAN, on tariff and non-tariff barriers. The term of non-tariff barrier encompasses a variety of government actions affecting trade. Sanitary and phytosanitary (SPS) is one of the non-tariff barriers which has been regulated by International Plant Protection Convention (IPPC). This is to prevent the spread and introduction of pests to plants and plant products, as well as to promote appropriate measures for their control.

Quarantine pests include fungal attacks, bacterial invasions, parasitism of nematodes and virus infection, or worst the combination and complex association of these pathogens. Diseases caused by plant pathogens, its rapid and wide dissemination via insect and nematode vectors, mechanical means and infected planting materials, and the ability of the virus genome to mutate and recombine, will lead to complexity of their detection.

Threats of pathogens are increasing as a result of globalization, human mobility, climate change, and others. Therefore, new technique is being developed almost on yearly basis due to constantly expanding discipline of plant virology. In term of market access, rapid test with high sensitivity of virus detection is needed to support phytosanitary regulations for international trading purposes. Technology transfer is needed to build capacity in the developing countries.

To directly address the devastating effects of plant diseases, a correct and reliable diagnosis is the ultimate pre-requisite. Diagnosis is the forefront of an efficient implementation of an effective disease management system. Aside from this, early diagnosis prevents possible entry and establishment of potential emerging pathogens from one to another country. To support market access, a common understanding and ability to apply the basic principles of phytosanitary measure is needed through capacity building.

The objective of the project on "Taxonomic capacity building to support market access for agricultural trade in the ASEAN region" coordinated by the ASEAN Plant Health Cooperation Network (APHCN) of the ASEAN Network on Taxonomy (ASEANET) and funded by Japan-ASEAN Integrated Fund (JAIF) is to develop and strengthen capacities in taxonomic knowledge to identify and manage quarantine risks associate with agriculture

commodities and to accurately diagnose pests and diseases among the ASEAN Member States (AMS). The first capacity building workshop was held in The Philippines on 17th -28th of August 2015 which involves representatives from Brunei Darussalam, Indonesia, Cambodia, Laos, Malaysia, Myanmar, The Philippines, Thailand, and Vietnam. Three participants were selected from the workshop to involve in this attachment program in Japan for two month from 26th October to 25th December 2015. The attachment program was held in Tokyo University of Agriculture (TUA). The three participants were from Indonesia, Malaysia and Vietnam.

Objective of attachment program

The objective of the attachment Program is taxonomic and capacity building to support market access for agricultural trade in the ASEAN region.

Daily Program

Day	Date	Activity
Monday	26th October 2015	- Arrival in Tokyo, move to Tokyo Nodai guest house, visit to the lab
Tuesday	27th October 2015	- Tour to the campus
		- Making phosphate buffer by using pH meter and electronic balance
Wednesday	28th October 2015	- Observation of various virus symptoms on diseased plants
		- ELISA for potyvirus from bamboo (*Pleioblastus chino*).
Thursday	29th October 2015	- Sawing of index plant (Passion fruit and bean) for virus inoculation
		- Inoculation of potyvirus into passion fruit seedling
Friday	30th October 2015	- Visit to Museum of Nodai

Monday	2nd November 2015	- Preparation of sample and conservation liquid
Tuesday	3rd November 2015	- National holiday
Wednesday	4th November 2015	- ELISA potyvirus from Passion fruit
Thursday	5th November 2015	- ELISA potyvirus from Passion fruit for second trial
Friday	6th November 2015	- Evaluation of ELISA and wrap up of the week
Saturday	7th November 2015	- Attending ISSAAS Congress
Sunday	8th November 2015	- Attending ISSAAS Congress
Monday	9th November 2015	- ISSAAS Excursion to Mount Fuji
Tuesday	10th November 2015	- Catch up holiday for 8th November 2015
Wednesday	11th November 2015	- Briefing and orientation - DNA extraction from BBTV infected banana and PCR
Thursday	12th November 2015	- Presentation on plant parasitic nematodes by Dr. Marita S. Pinili - Gel electrophoresis of BBTV DNA from banana
Friday	13th November 2015	- Extraction and detection of BBTV DNA from fresh and old banana samples as well as abaca samples - Post-lab discussion
Monday	16th November 2015	- Gel electrophoresis of BBTV PCR product from banana samples - Impregnation and extraction of virus nucleic acid from FTA plant card
Tuesday	17th November 2015	- PCR assay of DNA from FTA plant card - Electrophoresis, cutting gel, and purification DNA

Wednesday	18th November 2015	- Extraction of DNA BBTV directly from vector, *Pentalonia nigronervosa* - Ligation of purified DNA using pGEM vector
Thursday	19th November 2015	- DNA extraction from BBTV aphids impregnated on FTA card, PCR and gel electrophoresis - Transformation of ligated plasmid
Friday	20th November 2015	- Checking of colonies/transformants - Post-lab discussion - Observation of virus under electron microscope
Monday	23rd November 2015	- Picking transformed colonies of *Escherisia coli* and subculture on SOC medium
Tuesday	24th November 2015	- Mini preparation to extract plasmid from *E. coli* - Insert check using gel electrophoresis
Wednesday	25th November 2015	- Precipitation and processing sample for DNA sequencing
Thursday	26th November 2015	- Special lecture on DNA sequence analysis and phylogenetic by Dr. Noriko Furuya (DDBJ)
Friday	27th November 2015	- Analysis of sequencing result of BBTV
Monday	30th November 2015	- Discussion on the plan schedule of Yokohama trip and short visit to Utsunomiya University - Attending Halal Seminar by Nodai in cooperate with Putra Malaysia University
Tuesday	1st December 2015	- Preparation of LB medium for bacteria culture

Wednesday	2nd December 2015	- Aphid preparation for virus transmission: transfer aphid from healthy plant to infected plant and acquisition for overnight
Thursday	3rd December 2015	- Visit to Utsunomiya University to learn about dsRNA extraction from infected plant
Friday	4th December 2015	- Visit to Utsunomiya University to learn about dsRNA extraction from infected plant
Monday	7th December 2015	- RNA extraction for potyvirus from passion fruit (inoculated plant) using phenol chloroform method - Using reverse trancriptase to generate DNA from RNA
Tuesday	8th December 2015	- PCR and electrophoresis to extract DNA
Wednesday	9th December 2015	- DNA visualization under gel documentation
Thursday	10th December 2015	- Repeat of PCR process due to poor quality of DNA extraction - DNA visualization for check the DNA band - Preparation ofchemical chloride for TB medium
Friday	11th December 2015	- Visit to Yokohama Plant Quarantine Station and Research Center
Monday	14th December 2015	- Gel electrophoresis, DNA visualization - DNA purification
Tuesday	15th December 2015	- DNA ligation to pGEM vector - Detection of protein using SDS-PAGE and western blot
Wednesday	16th December 2015	- DNA transformation to *E. coli*
Thursday	17th December 2015	- Purification dsRNA

Friday	18th December 2015	- Attend PhD thesis defense from Ayaka Uke
Monday	21st December 2015	- Final presentation of attachment program report by three ASEAN participants (Indonesia, Malaysia and Vietnam) - DNA ligation to pGem vector
Tuesday	22nd December 2015	- DNA ligation to pGEM vector and DNA transformation into *E. coli* - Subculture transformant bacteria on LB medium - Visit Biomolecular laboratory for introduction to the illumina sequencing machine
Wednesday	23st December 2015	- Observation of *E. coli* colony growth on LB media. Culture with white colony showed successful transformation of DNA into *E. coli.* Unsuccessful transformation showed in blue colony.
Thursday	24nd December 2015	- Mini preparation to extract plasmid from bacteria - Move to hotel in Narita
Friday	25th December 2015	- Departure from Tokyo to Indonesia

PROGRAM ACTIVITIES

Laboratory experiments and activities during the attachment program.

<u>Laboratory activities assisted by Dr. Hogoken's students</u>

1. Buffer preparation for ELISA test and virus transmission

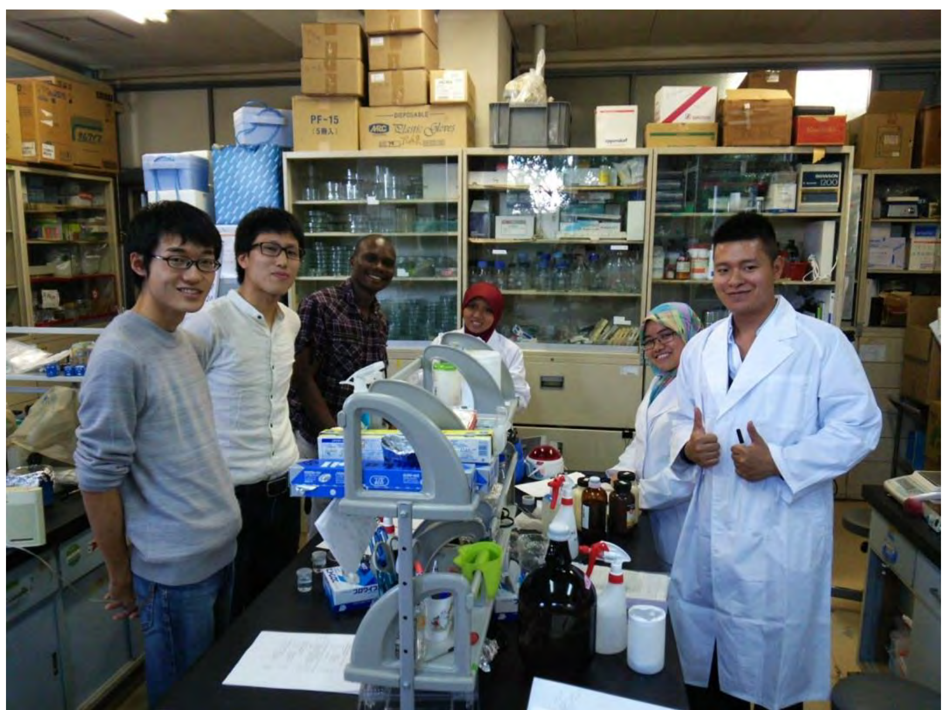


Fig. 1 First laboratory activity, preparation of buffer for ELISA test and virus transmission

2. Observation of virus symptoms on selected host plants

   Virus symptoms on plants observation were carried out at Nodai area. The diseased plants with symptoms includes Yabu myoga (*Polia japonica*), an ornamental plant of Japan caused by *Cucumber Mosaic Virus* (CMV), mosaic virus on taro (*Colocasia esculenta*) caused by potyvirus and mosaic virus on bamboo (*Pleioblastus chino*). Bamboo is one of the important commodity plants in Japan. The best known bamboo virus is Bamboo Mosaic Virus (BaMV) which belongs to the genus of *Potexvirus*. It causes mosaic on bamboo leaves and brown streak inside the shoots. This virus is found in all part of the plant and it is mechanically transmitted from plant to plant (possibly by aphid in the wild). Different with common BaMV, the BaMV found in *Pleioblastus chino* in Japan is remained little known. The main characteristic of this potyvirus is flexuous filamentous particle. Definitive potyvirus are aphid transmitted in a non-persistent manner and by sap inoculation.

Fig. 2 Field observation of virus symptoms on selected plants

3. ELISA test for potyvirus from selected plants samples

   ELISA is a serology method to detect virus by protein binding (antibody and antigen). In this experiment, bamboo (*Pleioblastus chino*) and taro (*Colocasia esculenta*) with virus infection symptoms were collected for the detection of potyvirus using indirect ELISA (Agdia protocol).

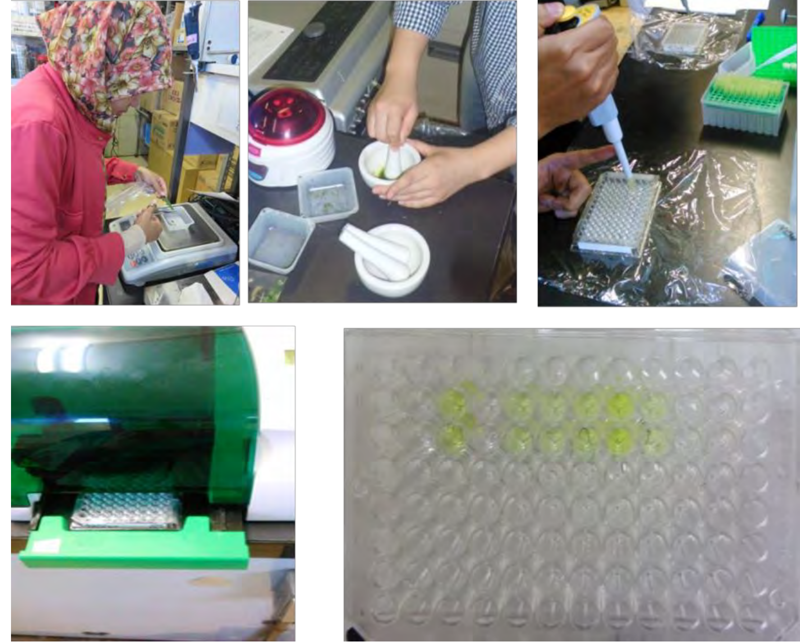


Fig. 3 Potyvirus detection using indirect ELISA method

4. Preparation of plant seedling for virus transmission

The activities started with planting host plants by sawing plant seeds into polybag. The plants were kept for seedlings grow. This was followed by mosaic symptom observation on infected Passion fruit's leaves. The leaves were kept for mechanical inoculation.

Fig. 4 Preparation of plant seedling for virus transmission

5. Virus transmission by using mechanical inoculation

Before preparing for virus transmission, the infected leaf sample was grinded using liquid nitrogen and followed by phosphate buffer. A drop of carborundum (Silicon carbide) was used to make hole on the leaf surface of the healthy plant seedling. The virus was transmitted to the healthy plant by rubbing the extraction onto the injured leaf surface. The plant seedling was inoculated in the growth chamber for 1-2 weeks for virus symptom observation.

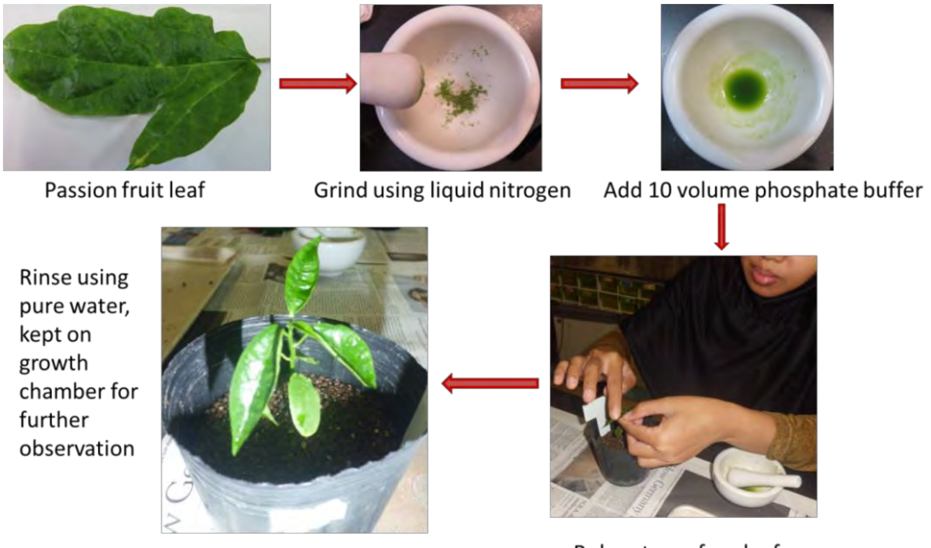


Fig. 5 Process of virus transmission using mechanical inoculation

6. Detection of virus using ELISA method

   After two weeks, the mosaic symptom was observed on the plant. The result was evaluated by indirect ELISA method for potyvirus target.

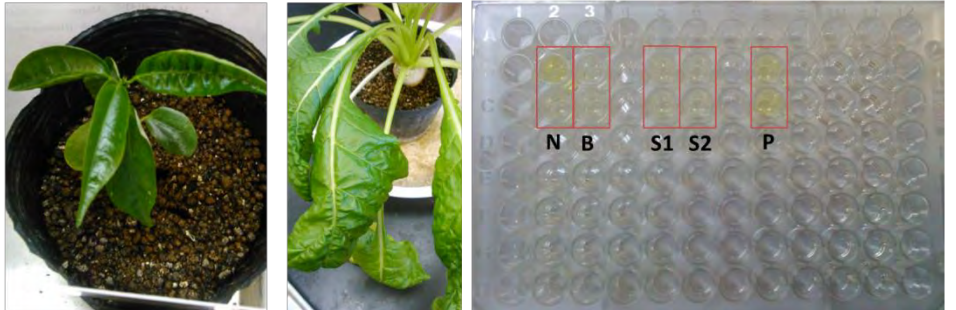


Fig. 6 Symptom observation after 2 weeks and evaluate with indirect ELISA for potyvirus

7. Detection of *Banana bunchy top virus* (BBTV) from fresh and old banana samples, aphid and FTA card using PCR method.

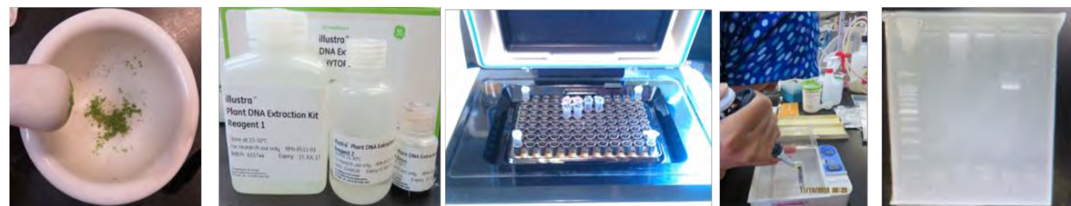


Fig 7. DNA extraction, PCR, and DNA visualization on gel agarose

Banana bunchy top disease (BBTV) is one of the most devastating viral diseases of banana (*Musa* spp.). It became a serious disease in many banana producing countries

in Asia and South Pacific. This experiment was to detect BBTV virus from fresh banana sample from laboratory, storted sample, aphids and FTA card by using PCR method. As a result, all DNA of BBTV were successfully isolated from the samples. The experiment was continuing with DNA cloning and sequencing. The DNA sequencing result was then analyzed using Basic Local Alignment Search Tool (BLAST) from NCBI. The result showed 98% similarity with BBTV DNA data from NCBI.

Fig 8. Impregnation sample (plant and aphid on FTA card)

Fig 9. Cutting gel, DNA purification, and cloning process

Fig 10. Loading DNA sample into plate for DNA sequencing process using sequencer machine

8. Virus observation under electron microscope (EM)

   Virus observation with Electron Microscope (EM) was one of the activities carried out during the attachment program. Before the sample can be observed under EM, virus sample is needed to undergo complex EM preparation protocol to help them withstand the environment inside the microscope. The sample was first grinded in PTA buffer. It was then stained with a thin layer of carbon coated with collodium membrane and covered with copper grid. The sample was ready to be observed under microscope. The result showed the characteristics of potyvirus make up of flexuous filamentous particle.

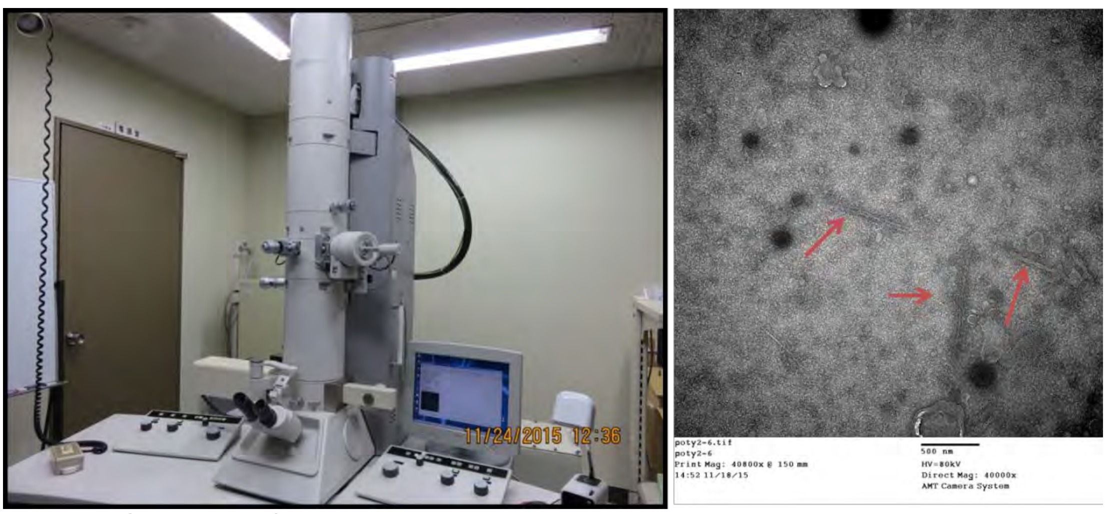


Fig 11. Observation of potyvirus under Electron Microscope (EM)

9. Extraction of dsRNA virus from plant to detect CMV from cucurbits

   The occurrence of high molecular weight double-stranded RNA (dsRNA) in plant is associated with the presence of dsRNA virus and replicating positive and negative sense RNA virus. Because dsRNA is very stable and can be easily extracted from relatively small amount of tissue, dsRNA profiling is an attractive tool for a preliminary diagnosis or characterization of viruses. This technique is based on interpretation of dsRNA banding pattern, different group of plant viruses have characteristics dsRNA pattern, the uniqueness of a profile based on the number and molecular weights of the dsRNA segment. For example, *Cucumber mosaic virus* (CMV) is a tripartite virus. The CMV genome consists of three single-stranded, messenger-sense RNA molecules, designated RNA 1 (~3,350 nucleotides), RNA 2 (~3,050 nucleotides) and RNA 3 (~2,200 nucleotides). The RNA 3 particle may contain a fourth RNA strand, referred to

as RNA 4 (~1,030 nucleotides), which encodes the coat protein gene and from which the CMV coat protein is produced

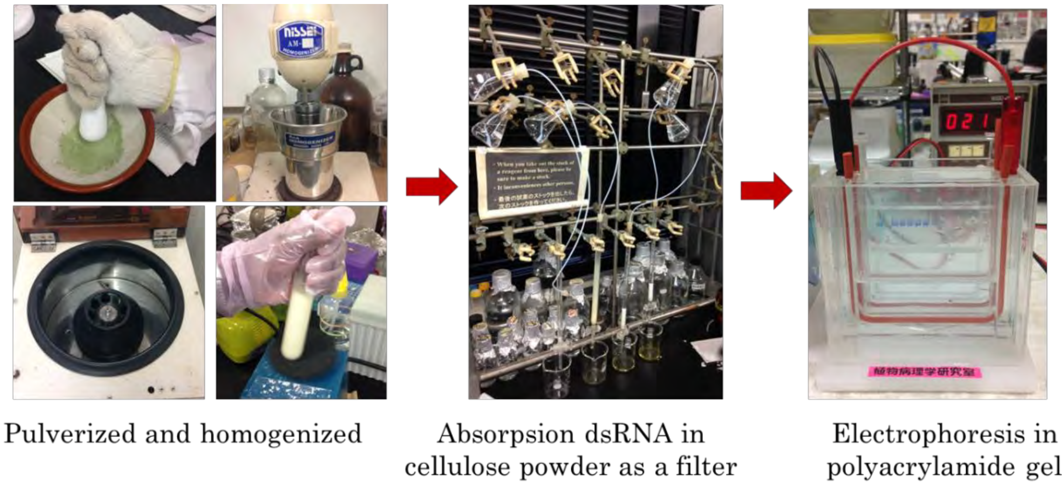


Fig 12. dsRNA extraction from cucurbits infected by CMV

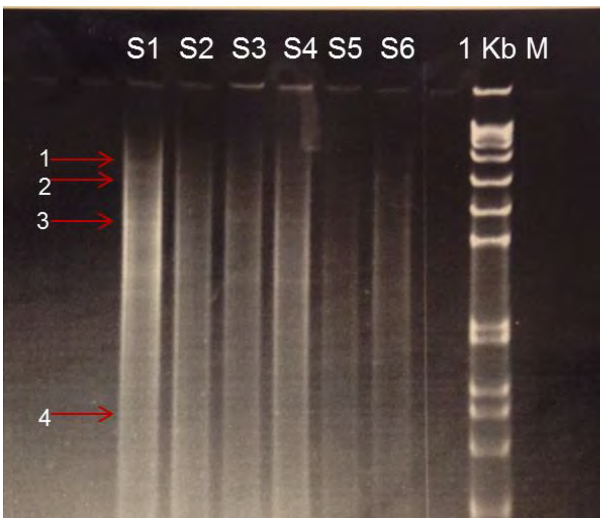


Fig 13. CMV dsRNA banding pattern have 3 main genome and one sub genome encoded coat protein

10. Detection of potyvirus from inoculated plant using phenol-chloroform method

    Detection of potyvirus was done using sample from previous experiment of virus transmission. After one month of inoculation, plants were evaluated using PCR to detect the successful transmitted potyvirus. RNA extraction from plant using phenol chloroform method was carried out.

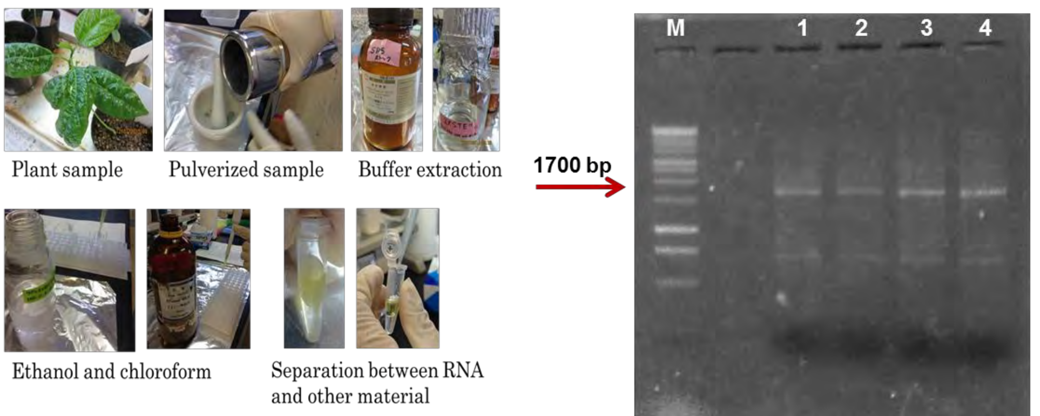


Fig 14. Extraction of RNA potyvirus from Passion fruit, and the result showed DNA band in 1700 bp compatible with DNA of potyvirus

11. Detection protein using SDS-PAGE method and Western Blot

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is a common method to separate proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. Protein separation by SDS-PAGE can be used to estimate relative molecular mass, to determine the relative abundance of major proteins in a sample, and to determine the distribution of proteins among fractions. The purity of protein samples can be assessed and the progress of a fractionation or purification procedure can be followed. Different staining methods can be used to detect rare proteins and to learn something about their biochemical properties. This training was conducted by Mr. Chong from NODAI.

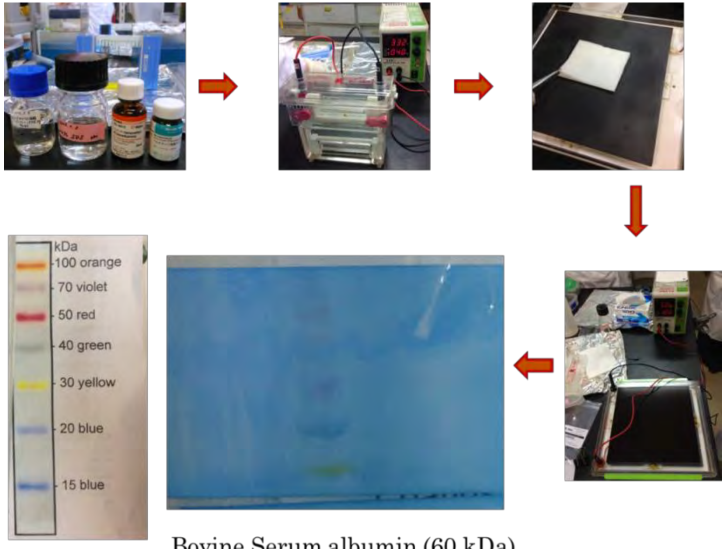


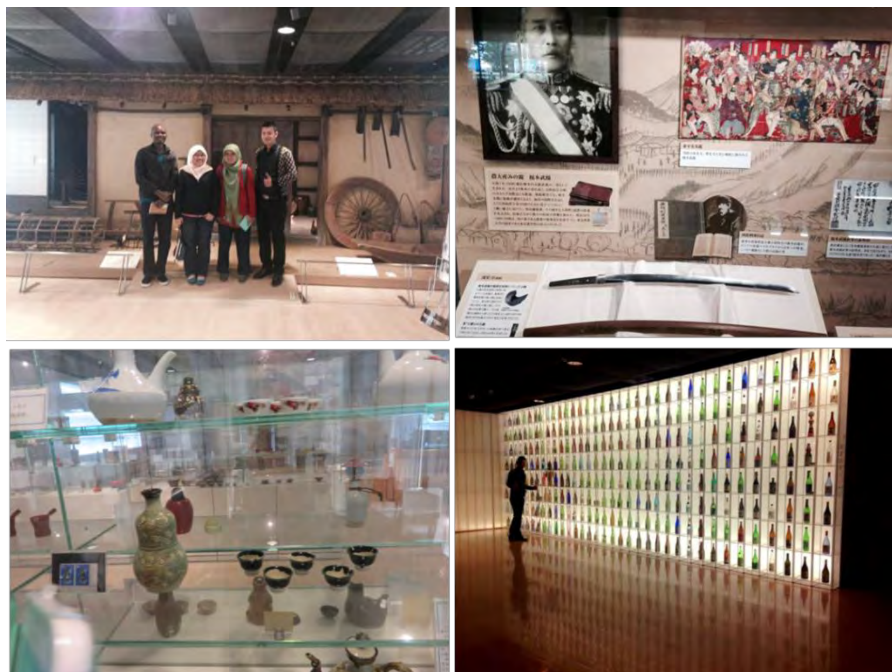
Fig 15. Process of SDS-PAGE and Western blot for sample of bovine serum albumin (BSA)

Other activities:

1. Harvest Festival of Tokyo Nodai (Shukaku-sai), flower arrangement, and tea ceremony

2. Attended International (ISSAAS) Congress that was held on 7 – 9 November at NODAI. Through the congress, I get to know more on the project development on agriculture science research.

3. Visited to Nodai Food and Agriculture Museum and Biorum



4. Attended lecture on attenuated virus by Dr. Keiko Natsuaki

   During the lecture, Dr Keiko Natsuaki showed us the video on plant cross protection technology in Japan. From the video, we get to know that the Japanese have great awareness on high quality agricultural commodity. Common use of the attenuated virus in Japan for cross protection is one of the strategy for biological control of plant viral disease. Cross protection appears to offer a promising strategy for biological control of plant viruses by produce virus resistant plant. Selection of cross-protective viruses from naturally occurring virus variants is generally done by observing infected plants in the field for mild symptoms, then culturing any isolated potentially mild virus strain. Cross protective virus variants have been obtained by growing virus infected plants at high (35°C) or low (15°C) temperatures. However, isolation of a mild viral strain is a labor-intensive and expensive process.

5. Attended lecture on nematode virus vector by Dr. Marita S. Pinili

6. Attended technical lecture on sequencing analysis and phylogenetic tree by Dr. Noriko Furuya from DNA Data Bank of Japan (DDBJ)

7. Attended Halal Food Seminar, New business for Japanese people. Hosted by Nodai and in cooperate with Putra Malaysia University

8. Visited to Utsunomiya University to learn about dsRNA extraction of virus

9. Visited to Yokohama Plant Protection Station to know about quarantine regulation in Japan

SUMMARY AND RECOMMENDATION

Diagnosis is the forefront of an efficient implementation of an effective disease management system. Aside from this, early diagnosis prevents possible entry and establishment of potential emerging pathogens from one to another country. To support market access, need a common understanding and ability to apply the basic principles of phytosanitary measure by capacity building.

The ability of diagnostic technique is very important to give further recommendation for pests and diseases. Recommendation can be pest controlling, pest management, and arrange regulation for international trade. This capacity building succeeds to open our mind about basic knowledge, advance technology and technical skills in the laboratory practices. Lessons, experiences, and knowledges sharing from experts, students, and colleagues are important to increase our competency about technical diagnostic for plant viruses. The lessons learnt would be very useful for our further work, especially for quarantine purposes to global market acceleration. For recommendation to the next project, I would like to get to know more on bioinformatics science. This is very a new area which is useful for many countries to characterize and identification of the pest, and the distribution of the pests.

ACKNOWLEDGEMENT

In this opportunity I would like to thank Japan-ASEAN Integrated Fund (JAIF), The ASEAN Plant Health Cooperation Network (APHCN), The ASEAN Network on Taxonomy (ASEANET), Dr. Lum Keng Yeang, and Dr Soetikno for giving us chance to attend this capacity building program. I would like to express my deeply appreciation to Dr. Marita S. Pinili as resource person, and my respectable Dr. Keiko T. Natsuaki from Tokyo University of Agriculture (Tokyo Nodai) as our training supervisor for their time and effort, guidance, sharing experiences, taking care our needs and accommodate us with comfortable accommodations and giving us advices. Last but not least, my friends and colleagues during my stay in Japan, thank you for making the training program exciting and full of fun.

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Appendix 1

Preparation of Phosphate Buffer and 5X PBST Buffer

(a) Preparation of 0.1 M Phosphate Buffer

Materials:

$Na_2HPO_4.12H_2O$ (sodium phosphate), FW=358.14

KH_2PO_4 (potassium phosphate), FW=136.69

Methods:

1. Sodium phosphate was weigh, 7.16 g and potassium phosphate, 2.72 g.
2. The chemicals were dissolved into 200 mL distilled water, repectively.
3. After that, adjust the pH of sodium phosphate solution with potassium phosphate solution until pH 7.0.
4. The solution was transferred into Schott bottle and properly labelled.
5. The solution was stored inside the chiller.

<u>Calibration of pH meter</u>

1. The pH meter was turned on and button [EXIT] was pushed.
2. The electrode tip was washed using pure water and wiped off with tissue.
3. Electrode tip was placed in standard pH solution, pH 9.18 (alkaline) and button [cal] was pushed.
4. The display value of standard pH was confirmed and saved.
5. Step 2 to 4 was repeated. This time, pH solution was changed to pH 6.8 (acid).

(b) Preparation of 5X PBST Buffer

Materials:

NaCl (sodium chloride)	20 g
$Na_2HPO_4.12H_2O$ (sodium phosphate)	2.875
KH_2PO_4 (potassium phosphate)	0.5 g
KCl (potassium chloride)	0.5 g
Tween 20	1.25 mL

Methods:

1. All the materials for PBST buffer were weigh according to the recipe.
2. The materials were dissolved in 400 mL of distilled water.
3. Then, the pH of solution was measured and adjusted using sodium hydroxide (NaOH), pH 7.4.
4. Distilled water was added into adjusted pH solution until reached 500 mL.
5. The solution was transferred into Schott bottle, labelled and stored inside the chiller.

Medium Composition and Preparation

SOC Medium ($MgCl_2$, $MgSO_4$, Amplicin): 50 mL

Bacto tryptone	1 g
Bacto yeast extract	0.25 g
NaCl	0.025 g
Pure water	40 mL + α
1 M glucose	1 mL
1 M $MgCl_2$	500 uL
1 M $MgSO_4$	500 uL
Amplicin solution	50 uL

- Glucose (1 M): 10 mL

Glucose 1.8 g
Pure water 10 mL
0.22 uM MILLIPORE filter
10 mL syringe

- $MgSO_4$ (1 M): 10 mL

$MgSO_4.7H_2O$ 2.46 g
Pure water 7 mL + α

- $MgCl_2$ (1 M): 10 mL

$MgCl_2.6H_2O$ 2.04 g
Pure water 7 mL + α

LB Medium (Ampicillin, IPTG, Xgal): 300 mL

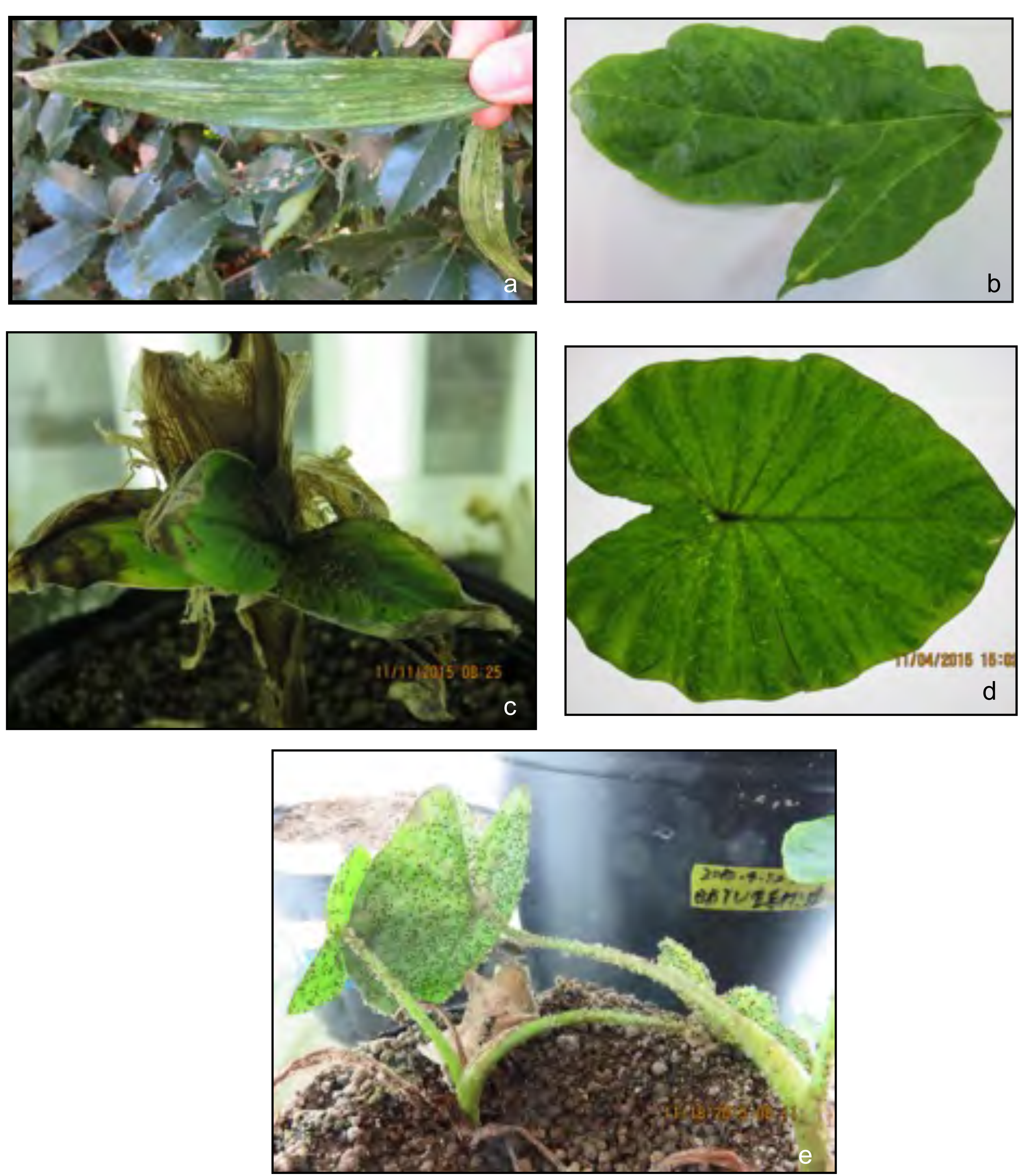
Bacto tryptone	3 g
Bacto yeast extract	1.5 g
NaCl	3 g
Agar	4.5 g
Pure water	200 mL + α
IPTG solution	300 uL
Xgal	300 uL
Ampicillin solution	300 uL

IPTG solution (1 M isopropylthio-β-galactosidase): 10 mL
IPTG (isopropylthio-β-galactosidase) 238 g
Pure water 10 mL
0.22 uM MILLIPORE filter
10 mL syringe

Xgal (2% 5-bromo-4-chloro-3-indole-β-D-galactoside): 5 mL
Xgal (5-bromo-4-chloro-3-indole-β-D-galactoside) 100 mg
0.22 uM MILLIPORE filter
10 mL syringe

Appendix 3

Symptoms observation on several samples



Leaves samples infected by viruses (a) Bamboo; (b) Passion fruit; (c) Banana; (d) Taro; (e) Taro leaves infested with aphid.

Appendix 4

Deeping Technique for Electron Microscope Samples Observation

Materials and methods

Preparation of negative stain reagent

Concentration to be prepared: 1% phosphotungstic acid (PTA)

1. The phosphatungstic acid was weigh, 1g for each student.
2. 50 mL of distilled water was added into the phosphatungstic acid and let it dissolved.
3. Optional: The pH of the solution can be adjusted using sodium hydroxide (NaOH).
4. The solution was stored at 2 - 8°C.

Preparation of virus sample

1. The glass slide was taken and washed with distilled water. The glass then dried out using tissue paper.
2. One drop of phosphatungstic acid (PTA) reagent was dropped on the glass slide.
3. The small size leaf sample was cut off. The sample should include leaf surface and vein. This is to ensure more virus sample taken and observed under the electron microscope later.
4. Then, the leaf sample was sliced and mixed with phosphatungstic acid to make a sap solution.
5. One disc of copper was touched on the sap solution for 1 to 2 sec. The disc was dried using filter paper and placed it back in the disc grid.

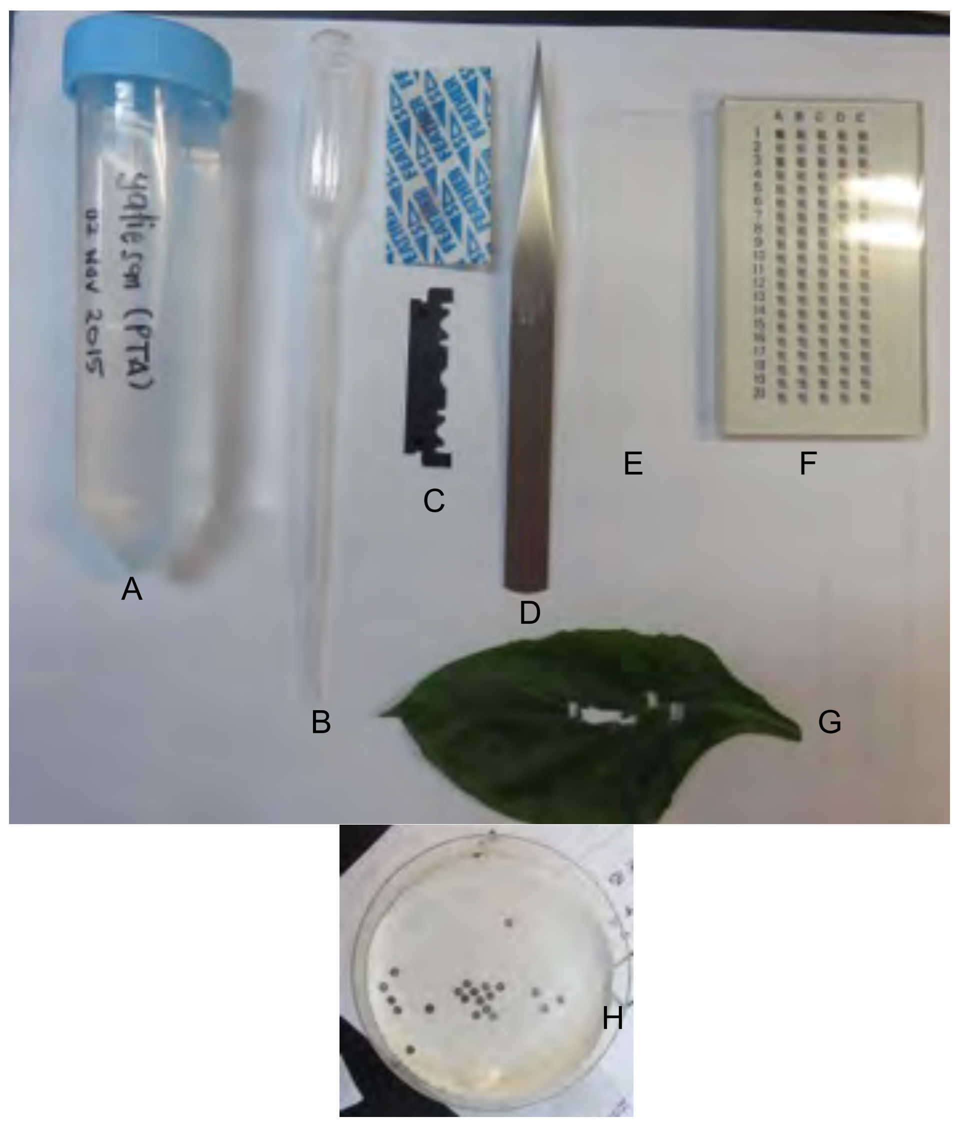


Figure 1: Materials used for the preparation of samples. (A) PTA reagent; (B) plastic dropper; (C) blades; (D) forcep; (E) glass slide; (F) disc grid; (G) leaf sample; (H) copper disc.

Figure 2: Preparation of sample using deep method.
(a) One drop of PTA reagent was dropped on the glass slide; (b) a small size of leaf sample was cut off including portion of vein; (c) the leaf was crushed and mixed with PTA reagent; (d) the copper disc was dried using filter paper; (e) the disc was placed back into the grid.

Appendix 5

Detection of Bamboo Virus (Potyviruses) Using Indirect ELISA

Introduction

Pleioblastus chino is typical chino bamboo which characterize as small, more narrow, have white stripes leaves with hairy on both sides. Chino bamboo is planted as attractive ornamental plants. Besides that, the plants are planted for many purposes such as containers, screen, hedge, house compartments, wood, crafts as well as edible shoots.

Same as other crops, chino bamboo also can be infected by viruses. One of the virus is Bamboo Mosaic Virus (BaMV). The virus is belongs to the Potexvirus, family of Alphaflexiviridae. BaMV is not transmitted by insect vector, however, the virus can be disseminate by mechanical or contamination of agriculture tools.

Besides that, there are some other viruses which show similar symptoms such as mosaic, chlorotic and necrotic streaking. However, detection and identification the specific spesies of the viruses still in progress. Researches have to find the main causal agent and vectors which could be responsible to the symptoms appear from the infection. The appeared symptoms also similar to the Sugarcane Mosaic Virus (ScMV). ScMV caused by single Potyvirus. After 2 decades, this virus were included in sugarcane mosaic virus group consisting 5 distinct species of potyviruses. There are sugarcane mosaic virus (SCMV), sorghum mosaic virus (SrMV), maize dwarf mosaic virus (MDMV), Johnson grass mosaic virus (JGMV) and zea mosaic virus (ZeMV). However, only SCMV and SrMV infects the sugarcane in natural environment. These viruses will transmistted in 3 different ways including by aphid vectors, by infected stalk cuttings and by mechanical inoculation.

Other unknown potyvirus has been reported on *Pleioblastus chino* in Japan. However, there is no scientific reports and less information about the virus, symptoms and their host range. Suspected virus has been found to cause seedling leaf stripping and stunting on *Bambusa bambos* in nursery seedlings. The virus spread systematically to the entire plant. If this infection by the virus can be proved, it could be consider as seed transmittable virus which spread at low concentration and then cause infection shortly after seed germination. Therefore, this experiment was conducted to detect the potyvirus infected bamboo using indirect ELISA.

Materials and Methods

1. Bamboo leaf samples (AZUMANE-Zasa – *Pleioblastus chino*) were taken from main gate (Figure 1) and Kyodo gate of NODAI (Figure 2). Another 3 samples (2 – from main gate on 27th Oct 2015); 1 – from Nakaniwa: Figure 3) were taken by student. Samples was showed mosaic symptom on the leaf. The samples were brought to the laboratory for ELISA analysis.

2. The samples were weigh 0.1 g each and crushed using liquid nitrogen. The grinded samples were mixed with 1 mL extraction buffer and transferred into 1.5 mL of microcentrifuge tubes.

3. The samples were centrifuged for 5 min at 15,000 rpm.

4. Supernatant of each samples (100 µL) were transferred into each well of microplates.

5. The microplates were incubated for 1 hour.

6. Towards the end of incubation time, 1X PBST buffer was prepared to wash the microplates.

   Preparation of 1X PBST Buffer:

   $M_1V_1 = M_2V_2$

   $(5)V_1 = (1)(1000)$

   $V_1 = 200$ mL

   Mix 200 mL of 5X PBST buffer with 800 mL distilled water.

7. Microplates were washed for 3 times using microplate washer. Microplates were dried out again using paper towel to ensure all the remaining washing buffer out from the wells.

8. Then, antibody was prepared and dispensed into each well (100 µL).

   Preparation of antibody:

   1: 200

   1700 µL (for 16 wells) = x

   X = 8.5 µL antibody

   Therefore, 8.5 µL of antibody diluted with 1700 µL ECI Buffer

9. Microplates were incubated for 2 hours.

10. After incubation, step 7 was repeated.

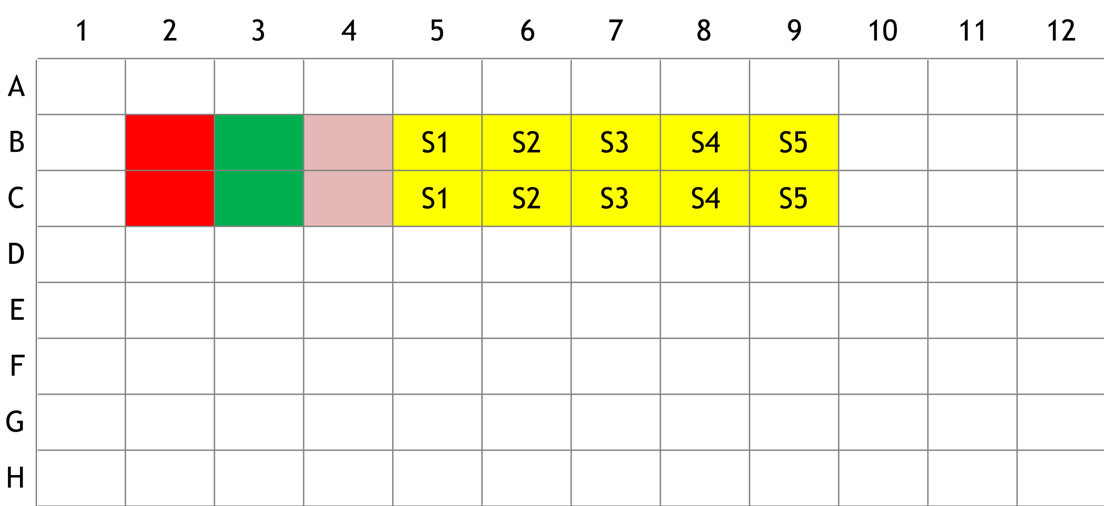
11. Enzyme conjugate was added into each wells (100 µL). Enzyme conjugate was prepared with same volume of antibody.
12. Microplates were incubated again for 1 hour.
13. Step 7 was repeated.
14. Each wells was added with PNP substrate (100 µL)

    Preparation of PNP substrate:

    1 PNP tablet (5 mg) was mixed with 5 mL PNP solution

15. The microplates were incubated in the dark for 1 hours. The results were evaluated and recorded every 15 min.

<u>Microplate Layout</u>



	1	2	3	4	5	6	7	8	9	10	11	12
A												
B					S1	S2	S3	S4	S5			
C					S1	S2	S3	S4	S5			
D												
E												
F												
G												
H												

Notes:

Results and Discussion

Samples	ELISA Readings							
	15 min		30 min		45 min		60 min	
Negative control	0.071		0.076		0.082		0.082	
Positive control	0.268		0.506		0.729		0.919	
Buffer	0.066	-	0.066	-	0.066	-	0.066	-
S1	0.521	+	1.014	+	1.441	+	1.819	+
S2	0.377	+	0.698	+	0.993	+	1.269	+
S3	0.865	+	1.708	+	2.415	+	3.047	+
S4	0.067	-	0.071	-	0.074	-	0.078	-
S5	0.196	+	0.351	+	0.493	+	0.627	+

Based on the results, 4 bamboo leaf samples (S1, S2, S3 and S5) were showed positive detection of potyvirus and the readings were consistently positive within 1 hour incubation period. All the positive potyvirus were showed mosaic symptoms and yellow streaking. The mosaic patterns was run parallel with the leaves vein. However, only S4 showed negative result on potyvirus (low absorbance values). This situation was happened due to the target protein is not expressed or low level of target protein expressed in samples. To overcome this situation, the amount of sample used should be increased.

Additional Activities

Objective of this activity was to observed the change of the ELISA reaction in different condition and amount of sodium hydroxide (NaOH).

ELISA Plate 1: Placed in freezer overnight, no additional NaOH.

ELISA Plate 2: Placed in room temperature, no additional NaOH.

ELISA Plate 3: Placed in room temperature with 50 µL NaOH.

ELISA Plate 4: Placed in room temperature with 100 µL NaOH.

Results

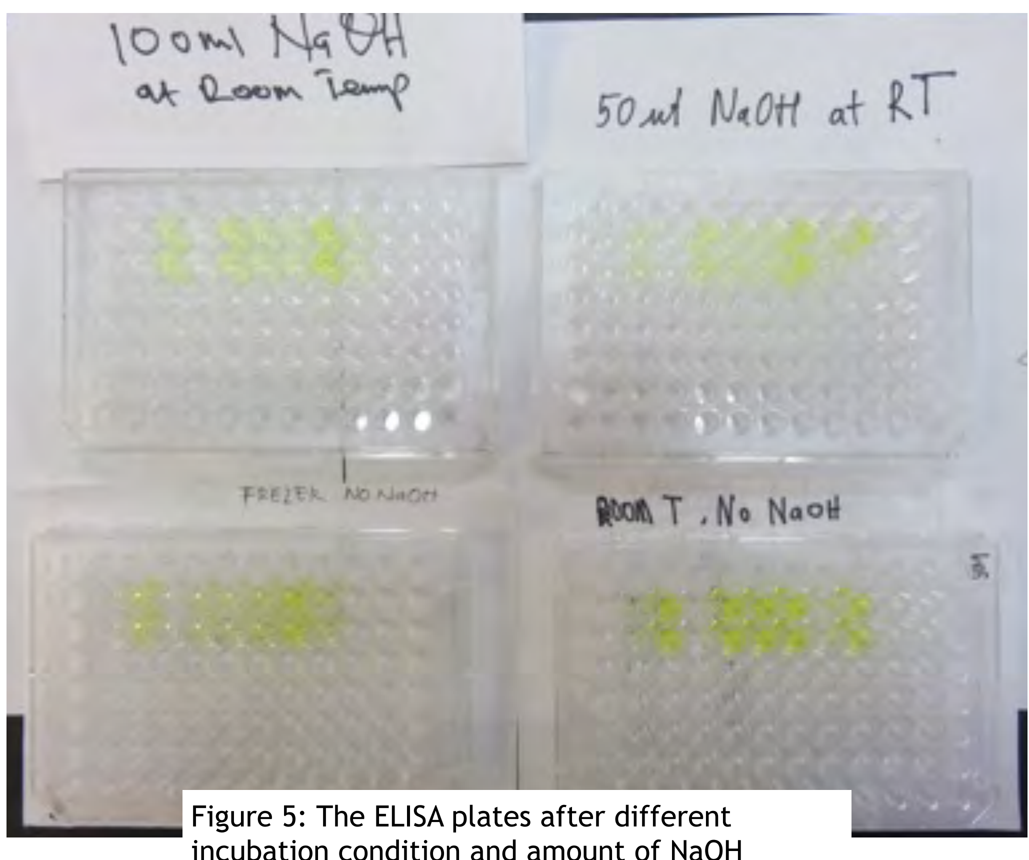


Figure 5: The ELISA plates after different incubation condition and amount of NaOH added.

Plates	Observation after incubation
1	The reaction was totally stop.
2	The reaction was not stopped and color of samples from well No. 8B and 8C is not changed (pale yellow) compare to other samples (strong yellow)
3	The reaction was stopped.
4	The reaction was stopped.

Discussion

Based on colour observation, the enzyme reaction from 3 of the ELISA plates (Plate 1, 2 and 3) were stopped. The enzyme reaction in plate 1 is totally stop due to the low temperature during incubation in the freezer. Low temperature will slow or stop any of the enzyme activities. For ELISA plate 2 and 3, NaOH was commonly used as stop buffer to stop ELISA reaction. However, in ELISA plate 4, different result was observed. Almost all of the samples was changed in colour from pale yellow to strong yellow. This indicates that the enzyme reaction was not stopped. Besides that, wells No. 8B and 8C showed pale yellow. This result was different compare to other wells due some reasons:

1. Mistakes in preparation of enzyme conjugate and substrate. Therefore, no reaction between those 2 solution to change the colour of the samples.
2. Pipetting error: Unequal volume of ELISA solution was transferred into the wells.

Conclusion

Leaves samples taken from the bamboo which showed typical mosaic symptoms were infected by Potyvirus. Besides that, some precaution should under taken during the ELISA test to ensure the it will produced reliable, accurate and precise results.

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Appendix 6

Detection of Taro Viruses (Potyviruses) using Indirect ELISA

Introduction

Taro (*Colocasia esculenta*) is belongs to family Araceae. The plant used as vegetables for their corms (thickened underground stem), leaf and leaf stems (petioles). Taro also called as darsheen or eddoe. Like other vegetable plants, taro can be infected by some plant viruses. The infection from viruses will cause severe yield reduction and plant death. Previous studies were reported that taro are susceptible to several plant viruses infection. The viruses are Darsheen Mosaic Virus (DSMV), Colocasia Bobone Disease Virus (CBDV), Taro Bacilliform Virus (TaBV) and Taro Vein Chlorosis Virus (TaVCV) (Babu et al., 2011).

a) Darsheen Mosaic Virus (DSMV)

This virus is belong to Potyvirus group. The infected plants were showed symptoms as feathering of tissue along the vein, severe slight vein banding or no visible symptom at all. Sometimes a single plant may have white or pale striking symptom on the leaves. The sizes and shapes of leaves also affected and low yield corm. The virus was transmitted by 3 ways; a) by insect vectors (Myzus perpicae, Aphis craccivora, Aphis gossypii) non-persistent manner; b) by vegetatively (suckers, corms or infected cuttings used for propagation); c) by mechanical (by plant sap on knives or shears). However, the virus was not transmitted by seed or pollen.

b) Colocasia Bobine Disease Virus (CBDV)

The virus was first reported by James et al. (1973). The virus was detected in the samples taken from Solomon Islands. The infected plants were showed as thickened malformed and brittle leaves and severe stunting. The virus was spreader by insect vector in persistent manner (Tarophagus proserpina). However, the virus was not transmitted by mechanical inoculation, contact between plants or by seed and pollen.

c) Taro Vein Chlorosis Virus (TaVCV)

The virus is belongs to the Rhabdoviridae group. The symptoms showed by infected plants were chlorosis of the veins, near the leaf margin, maximum growth after planting or at maturity, the leaves have district vein chlorosis which associated with TaBV (Taro

Bacilliform Virus). As the leaves aged, chlorosis spreads between the veins and form a network and become necrotic and leaf margins have a tattered appearance.

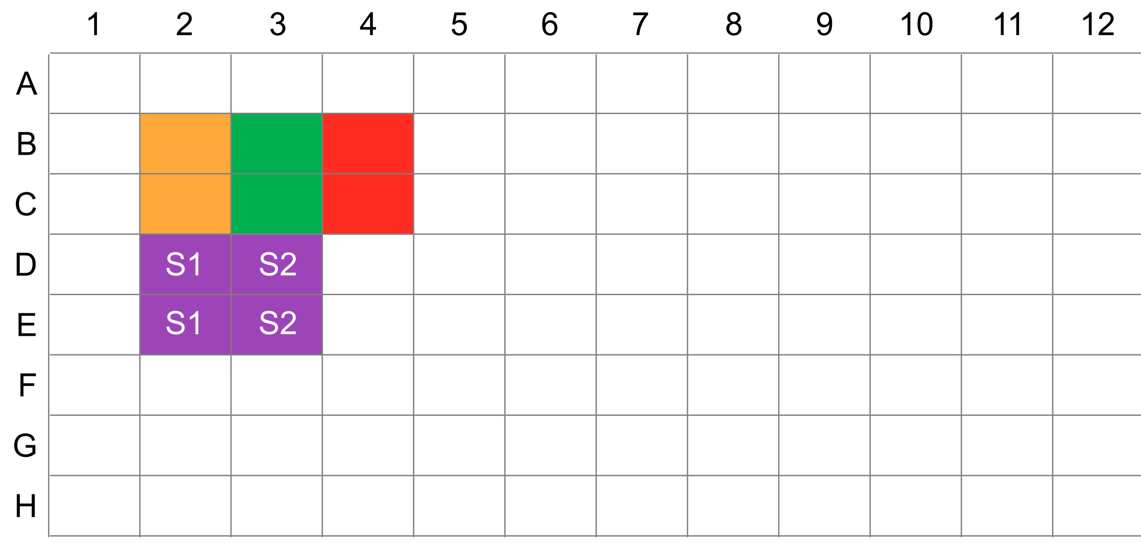
The objective of this experiment was to detect the virus (Potyvirus) which infect the taro plants.

Materials and Methods

1. Taro leaf samples were taken were taken by student. Samples was showed mosaic symptom on the leaf. The samples were brought to the laboratory for ELISA analysis.
2. The samples were weigh 0.1 g each and were mixed with 1 mL extraction buffer before crush and transferred into 1.5 mL of microcentrifuge tubes.
3. The samples were centrifuged for 5 min at 15,000 rpm.
4. Supernatant of each samples (100 µL) were transferred into each well of microplates.
5. The microplates were incubated for 1 hour.
6. Microplates were washed for 3 times using microplate washer. Microplates were dried out again using paper towel to ensure all the remaining washing buffer out from the wells.
7. Then, antibody was prepared and dispensed into each well (100 µL).
8. Microplates were incubated for 2 hours.
1. After incubation, step 7 was repeated.
2. Enzyme conjugate was added into each wells (100 µL). Enzyme conjugate was prepared with same volume of antibody.
3. Microplates were incubated again for 1 hour.
4. Step 7 was repeated.
5. Each wells was added with PNP substrate (100 µL)
6. The microplates were incubated in the dark for 1 hours. The results were evaluated and recorded every 15 min.

Figure 1:
Infected taro leaves showed mosaic symptom. (a) T808 T254P; (b) T706 T19

Microplane Layout



Notes

(red)	Negative control (healthy taro leaves)
(green)	Positive control
(orange)	Buffer
S1	Taro leaf (7808 T254P)
S2	Taro leaf(T706 T19)

Results and Discussion

Samples	ELISA Readings							
	15 min		30 min		45 min		60 min	
Negative control	0.358		0.613		0.850		-	
Positive control	2.569		****		****		-	
Buffer	0.070	-	0.073	-	0.074	-	-	
S1	0.9265	+	1.738	+	2.380	+	-	
S2	3.413	+	****	+	****	+	-	

Note:

**** - Strong yellow: the absorbance is too high

Based on the result showed in the table below, both taro leaves samples were showed positive detection of Potyvirus using indirect ELISA. The absorbance readings were consistently positive after 45 mins for the samples and control. However, the absorbance readings were only recorded until 45 mins because the positive control and S2 sample showed strong yellow colour and no readings stated by the ELISA reader. This situation happened due to the high concentration of control and sample and its is out of range for the sensitivity of the assay. To overcome the problem, re-assay should be done or reduced the concentration of sample and control by dilution before adding into the wells. The dilution factor should be took into account when in the results calculation.

Conclusion

Both taro leaf samples taken which showed typical mosaic symptoms were infected by Potyvirus. Besides that, re-assay should be done to get an accurate results on positive control and samples. PCR can be the most suitable technique can be used to identify the species of Potyvirus which infect the taro plants.

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Appendix 7

Inoculation of Unknown Passion Fruits

Introduction

Passion fruit (*Passiflora* spp.) was planted in temperate area all over the world. Same as other crops, passion fruit has been reported to be infected with insect pest and diseases caused by bacteria, fungi and as well as viruses. According the recent researchers, there are 10 important viruses were infected the fruit. There are:

a) <u>Potyviruses diseases</u>

First infection of potyvirus on passion fruit was discovered in Australia (PMV: Passion fruit woodiness virus), followed by Nigeria (1962), Taiwan (1992) and Japan (1996). However, the potyvirus which infects Brazil was different compared to other countries. The virus strain was identified as cowpea aphid-borne mosaic virus (CABMV).

Symptoms:

The associated infection by 2 strains (PMV and CABMV) were appeared as severe mosaic, rugosity, distortion of leaves, a reduction of plant development and woody and deformed fruits. Besides that, the symptoms caused by associated infection between PMV and sugarcane mosaic virus (SMV) was showed as severe mosaic, epinasty, defoliation and premature death of plants. Infected passion fruit flowers will become mottled an ringspot on the younger leaves. The infected fruit will appear as symptomless. Other plant viruses involved in passion fruit infection are passion fruit mottle virus (PaMV) which induce skin mottling on fruits, passiflora virus Y appear as mottling on leaves and east asian passiflora virus (EAPV) caused chlorotic spots on leaves and faded fruits.

Mode of transmission:

The viruses can be transmitted by aphids including Myzus persicae, Aphis gossypii, Aphis spiraecola and Totoptera citricidus in non-persistent and non-circulative manner. In addition, the viruses also transmitted by mechanical grafting. The potyviruses infest wide host range such as Passifloraceae, Fabaceae (Leguminose), Nicotina benthamiana, Nicotina clevelandii, Nicotina tabacum (solanaceae), Chenopodiceae (Chenopodium album, Chenopodium amaranticolor and Chenopodium quinoa) and Gomphrena globosa

(Amaranthaceae). Thus, the systemic infection will caused development of symptomatic or latent on the fruit.

b) Cucumber mosaic virus (CMV)

CMV have single stranded RNA genome consisting unique RNAs 1, 2 and 3 which encapsidated separately in same capsid protein. The infection by the virus only occured with the presence of these 3 RNAs. The virus can be transmitted by aphids (non-persistent) and through mechanical practices (commonly through seedlings).

c) Passiflora latent virus (PLV)

The virus is belongs to the genus of carlavirus. The virus has characterize to have filamentous flexous rods and consists of ssRNA. The infection of virus was first reported in Germany which infect Passiflora caerulea and Passiflora suberosa). The symptoms will appear as inconspicous and systemic foliar mosaic. In cooler weather, the older leaves shows mottled. Mode of transmission of the virus is by aphids in non-persistent and non-circulative way.

d) Passion fruit yellow mosaic virus (PaYMV)

The virus is belongs to the genus of Tymovirus. The infection by PaYMV was reported only in Brazil and Colombia. The symptoms show as bright yellow mosaic, yellow met and crinkled leaves. PaYMV transmitted into the plants only by mechanical way. However, the virus will not transmitted by seeds. Recently, the virus particles of PaYMV was transferred into passion flower in Brazil by Diabrotica speciosa (bettle).

e) Passion fruit vein clearing virus

The baciliform-like particles (particularly from genus Rhabdovirus) was reported to cause passion flower vein clearing disease. The symptoms appear as vein clearing, severe yield loss and size reduction in fruits and leaves. The virus transmits through plant grafting.

f) Purple grandilla mosaic virus (PGMV)

This type of virus was found in Passional edulis in Brasil. The infected plant was showed exhibit mild or line pattern mosaic on leaves. The fruits become smaller in size, deformed and woody. The virus was transmitted by beetle, Diabrotica speciosa.

g) Passion fruit green spot virus (PGSV)

The green spots with 2.5 mm diameter on mature yellow fruits. Its shows uniformly green with central necrotic depression, in isolated patches on senescent, chlorotic leaves along the veins.

h) Geminivirus disease

Passiflora leaf mottle virus transmitted by Bemisia tabaci. The symptoms showed as severe curling, distortion, mottling leaves, low quality of fruits and decrease the yield production.

Passion flower little leaf mosaic virus (PLLMV) infect passion flower will show exhibited intense yellow mosaic on leaves. Severe infection will cause drastic reduction of leaf lamina.

i) Marajuca mosaic virus (MarMV)

The Tobamovirus was infected Passiflora eludes with symptoms appeared as leaf mosaic and crinkled. The virus was transmitted by contact with other plants and mechanical damage.

j) Tomato ringspot virus (ToRSV)

The virus was transmitted by Xiphinema americanum which belongs to Nepovirus.

Materials and methods

(A) Activity at HOGOKEN greenhouse

1. Virus infected passion fruit originated from different location was observed. The plants may infected by different viruses. Photos was taken.
2. Some infected leaves was taken for inoculation.
3. Two varieties of French beans and passion fruit seeds as test plant of passion fruit virus.
4. The pots were filled up with soil and brought back to the lab for sowing the seeds. Basically, 3 to 5 seeds were sow per pot (9 cm diameter). The labelled was placed in the pot to show the date of sowing, name of seed and student name.

(B) Activity at HOGOKEN laboratory

Materials

- Mortar pestles taken from freezing container of the fridge.
- Phosphate buffer.
- Carborundum powder.
- Fresh leaf samples as inoculation source.
- Passion fruit seedlings (one pot person) to be inoculated.

Methods

1. The passion fruit plan pots was set with label showing student's name and date of collection.
2. Weigh of fresh samples were measured (0.1 g).
3. Ten volumes/weight of sample was added with phosphate buffer.
4. Leaves were macerated to make sap juice.
5. Carborundum was mixed with the same and rubbed on the leaves tenderly.
6. After 3 - 6 min, extra sap was washed away by tap water only gently.
7. Mortar and pestle was washed by water and detergent and autoclaved or boiled them in boiling water for 10 min. The purpose is to inactivate TMV and RYMV. The used mortal and pestles were placed separately with other clean ones until they autoclaved/ boiled.
8. Keep the clean mortar and pestle in incubator for further observation.

Figure 1: Passion fruit plant infected with unknown viruses at glasshouse

Figure 2: Seed used for inoculation. (a) passion fruit; (b) V1 - French bean (pink seed); (c) V2 - French bean (black seed)

Figure 3:
Sowing of seeds

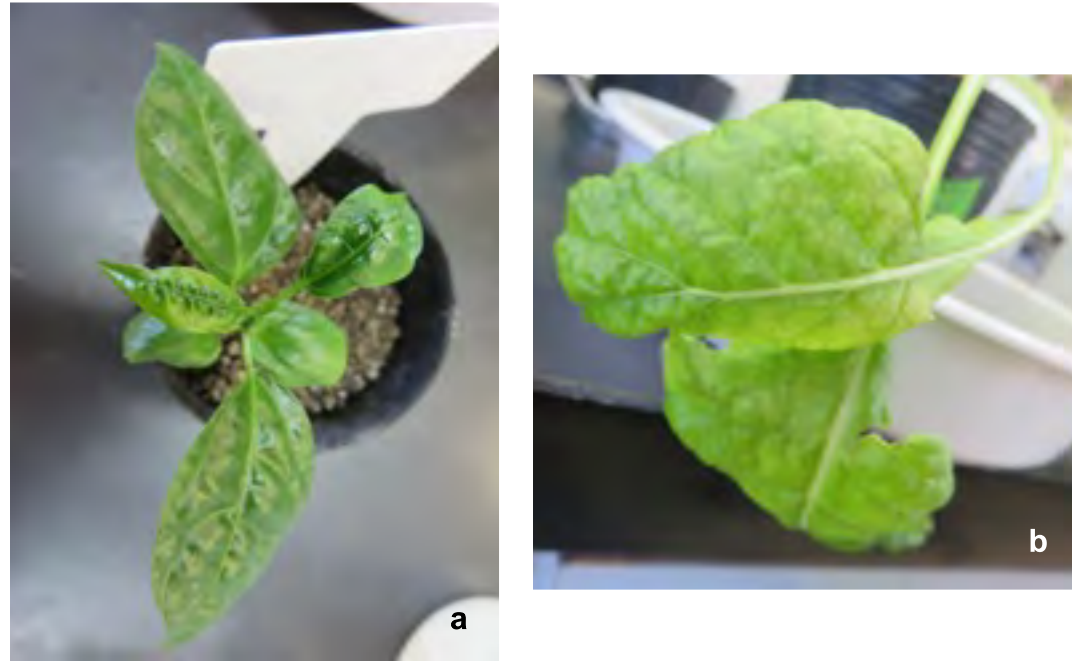
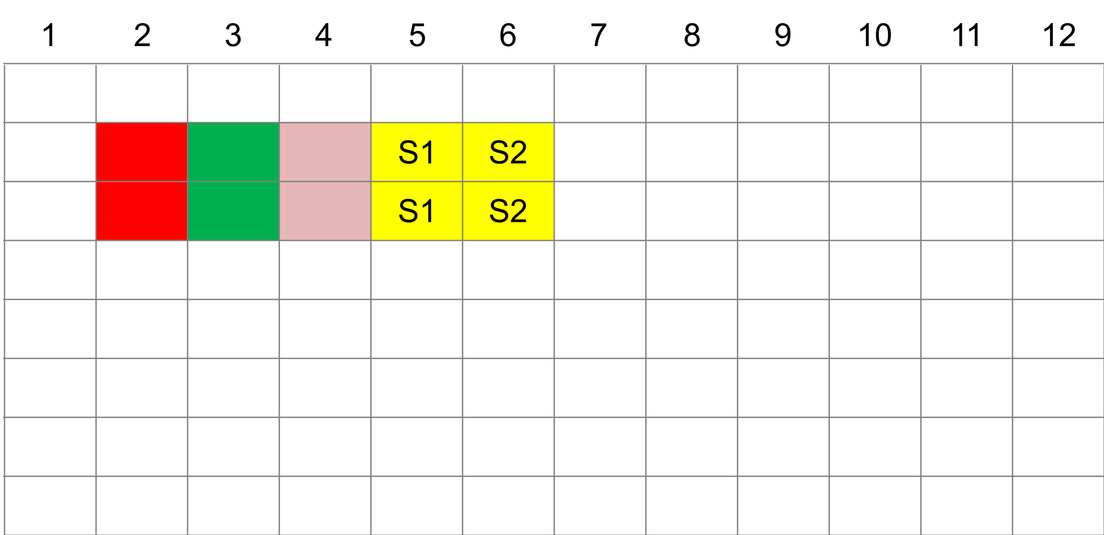


Figure 4: Plant samples used for ELISA test. (a) passion fruit inoculated with unknown virus; (b) reddish inoculated virus.

Microplate Layout

Note:



	Negative control (healthy passion fruit)
	Positive control
	Buffer
S1	Infected reddish leaf
S2	Sap inoculation passion leaf

Results and Discussion

Samples	ELISA Readings							
	15 min		30 min		45 min		60 min	
Negative control	0.075		0.082		0.090		0.097	
Positive control	0.075		0.077		0.080		0.084	
Buffer	0.070	-	0.072	-	0.075	-	0.077	-
S1	0.075	-	0.082	-	0.090	-	0.097	-
S2	0.075	+	0.202	+	0.279	+	0.344	+

Based on the result table above, sample S2 (sap inoculated passion fruit) was showed positive detection of potyvirus. However, Sample S1 (reddish) was showed negative detection of potyvirus. The reddish plant was inoculated with the same virus and the symptom showed as mosaic, rugose and yellowing of leaves. The negative result was obtained from the sample S1 because the concentration of virus accumulated in the plant was low compared to the sample S2. In addition, the low concentration of virus detected in the plant due to the less period of inoculation. The reddish plant was inoculated with virus less than a week. Therefore, less virus particles not evenly distributed to the whole of the plant compared to the passion fruit. The passion fruit was inoculated for a week and the concentration of virus particles should be more higher than reddish plant. To overcome this situation, the tested plant should be:

a) Inoculated with the unknown virus for at least 1 week before any ELISA test was conducted to detect the viruses.

b) Make sure the leaf samples choose for the test shows virus infected symptoms, unless the detection of viruses will not be successful.

c) Make sure the antibody, enzyme conjugate and substrate solution are added into the wells immediately to prevent the over dried of wells. The over dried of wells will affect the contact of reagent with virus particles and contribute to the negative result on test samples.

Conclusion

The sap inoculation of passion fruit was successfully conducted. The passion fruit was detected to be infected by the group of Potyvirus. The further study should be conducted to identify the specific potyvirus species.

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Appendix 8

DNA Extraction, PCR Amplification and Gel Electrophoresis of Banana bunchy top virus (BBTV) from Infected Banana

Introduction

Banana bunchy top virus (BBTV) was first reported cause infection on *Musa* spp. from Fiji (Magee, 1953). The virus was widespread in South East Asia including Philippines, Taiwan, most of South Pacific Islands and parts of India and Africa. Until now, there is no cure was applied to control the spread of BBTV infection. In addition, no varieties of banana are resistant to this virus. The infected banana shows symptoms rarely bear fruit because they are reservoirs of virus. Once the symptoms appear, the plant must be destroyed immediately. BBTV was transmitted by insect vector, aphid (*Pentalonia nigronervosa*) in persistent manner. The virus can be retained when the vector moults and its not transmitted to the progeny of vector or by mechanical inoculation.

According to Nelson (2004), the study was described several symptoms of BBTV on banana, which are:

a) Keikes: can be called ‘suckers’. The symptom develop after a ‘mother’ plant has been infected with BBTV and the plant is severely stunted. The leaves are not expand normally and remained bunched at the top of the pseudo stem. The infected leaves showed stiff and erect, shorter and narrower than normal leaves with chlorotic edges. The fruits will not showed the BBTV symptoms.
b) Maturing plants: new leaves will difficult to emerge properly, narrow than normal, wavy shape and have yellow (chlorotic) leaf margins. The plant appeared to be ‘bunched’ at the top. Severe infection by the virus will not appear. However, if fruit was produced, the banana hands and fingers will distorted and twisted.
c) Subtle disease symptoms: it requires close infection. The symptoms referred to ‘Morse Code Streaking’ and ‘ Green J-Hooks’.
d) Morse Code Streaking: initial symptoms consist of dark green streaks in the vein of lower portions of the leaf midrib and leaf petioles. The streaks also occur less prominent in the veins of the leaf lamina. The streak shapes are irregular and it resembles a series of dots and dashes. Observation of streaking symptoms more easier by rubbing away waxy white coating which covers the leaf petiole.
e) Green J-Hooks: the symptom appear as green-hook like extensions of the leaf lamina. The veins will seen in narrow with light green zone between the midrib and lamina.

Other symptoms showed as short hook point down along the midrib towards the petiole.

The objectives of the experiment were:

1. To extract DNA of BBTV from fresh and old samples of infected banana.
2. To detect and confirm the BBTV infection on banana samples using specific BBTV primers through PCR amplification.

Materials and method

1. Samples from infected banana were taken from the growth incubator. Then, 0.1 g of each samples were pulverised in liquid nitrogen. The pulverisation of sample was started with negative sample.
2. The well grained samples were transferred into the 1.5 mL micro centrifuge tube. 300 uL of plant DNA extraction Reagent 1 was added and turn the tube upside down or gently shake.
3. After that, 100 uL of DNA extraction Reagent 2 was added and once again turn the tube upside down or gently shake.
4. Step 1 to 3 was repeated for positive sample.
5. The samples were vortex for few second and heated for 10 min at 65°C using dry Thermo Unit.
6. Then, the samples were put in cold box for 20 min.
7. 250 uL of chloroform and shake gently.
8. 50 uL of resin was added. The resin was pre-vortex the resin to prevent the sedimentation.
9. The samples were centrifuged at 2500 rpm for 10 min at room temperature. The centrifugation should be repeated if plant debris are not completely settled.
10. First DNA was collected using 125 uL calibration and another 125 uL on the second (total DNA concentration is 250 uL).
11. All samples were added with 250 uL 2-propanol and shake gently.
12. The samples were centrifuged at 15000 rpm for 5 min at room temperature DNA template at the bottom of the tube.
13. 125 uL of supernatant was pipetted out. 100 all of 70% ethanol was added into the pellet.
14. The tubes were centrifuged at 15000 rpm for 2 min at room temperature.
15. The ethanol was pipetted out. The samples were dried out 2 to 3 min at room temperature.

Figure 1: Banana plant infected with Banana bunchy top virus (BBTV). (a)(b) Highly infested banana plant by aphid, Pentalonia nigronervosa; (c) Samples taken for DNA extraction: S1 and S2- unknown samples (positive or negative BBTV), S3, - healthy banana leaves, S4 - positive BBTV.

16. 100 uL of 1X TE buffer were added to the samples. The pellet was broke by touching with pipette tip.

PCR Protocol

Materials	For detection (uL)	For sequencing (uL)
q.s.	17.4	34.8
10X *Ex Taq* Buffer	2.5	5
dNTP mixture	2	4
Forward primer (usu. 25 pmol)	0.25	0.5
Reverse primer (usu. 25 pmol)	0.25	0.5
TaKaRa Ex Taq (5 units/uL)	0.1	0.2
Template cDNA	2.5	5
Total (uL)	25	50

Preparation of primers

2.5 uL of each primers (forward and reverse) taken from stock tube mixed with 75 uL 1X TE buffer. Total volume of each primers are 100 uL.

Procedures:

1. Cocktail mix was prepared and required amount was calculated (follow the sequence in adding chemicals as shown in the table).
2. Cocktail mix with 22.5 uL was dispensed into PCR tube.
3. DNA extract, 2.5 uL was added. PCR tubes were flashed for a few second to eliminate any bubbles.
4. The PCR machine was ran. After the cycles finished, samples were viewed by gel electrophoresis.
5. Precaution: *TaKaRa Ex Taq* should be placed in cold box. Do not touch the bottom of the tube with hands since it is temperature sensitive. Extra precaution should be taken in staining and de-staining the gel in ethidium bromide (EtBr). Gloves must be used.

Figure 2: Equipments used for the DNA extraction, PCR amplification and gel electrophoresis. (a) thermocycler; (b) centrifuge; (c) 1.5 mL micro centrifuge tube containing extracted DNA; (e) gel case and comb; (f) primers for BBTV; (g) UV illuminator with camera; (h) shaker; (i) containers containing EtBr and distilled water; (j) gel electrophoresis

Gel Electrophoresis

1. 2% gel was prepared.
2. Blue juice/loading dye was added, 2 uL on parafilm (droplets).
3. For 6 band-comb, 13 uL of PCR product was mixed into blue juice/loading dye by repeated pipetting. For 8 band-comb (small comb), 8 uL of PCR product used on 2 uL blue juice/loading dye.
4. The samples were loaded into gel starting with 100 bp ladder/marker (for BBTV) at 15 uL; followed by the negative sample.
5. The gel was loaded, the orientation should be from negative (-) to positive (+) at 100 V for 25 - 30 min.
6. The gel was stained in EtBr for 5 min.
7. Then, the gel was de-stained with distilled water.
8. Finally, the DNA bands were viewed under UV illumination and photo was taken using EDAS 290 (Kodak, Japan).

	Small gel	Big gel
Agarose powder (g)	0.25	0.5
1X TAE Buffer (mL)	12.5	25

Results and discussion

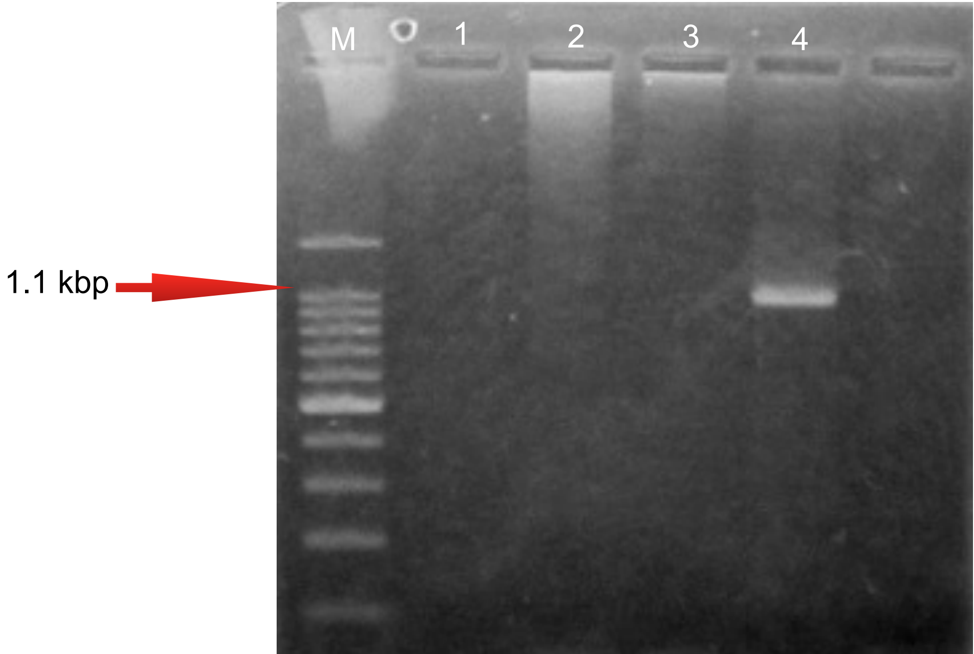

Figure 3: Gel electrophoresis of DNA extracted from banana leaves. The PCR products were analysed on 2% gel.

No. of well	Samples
M	100 bp ladder
1	S1 - unknown sample
2	S2 - unknown sample
3	S3 - healthy sample
4	S4 - infected with BBTV (positive sample)

According to Figure 3, DNA was successfully amplified from sample No. 4 (banana leaf infected by Banana bunchy top virus (BBTV). The length of DNA obtained using the PCR amplification was 1.1 kip which is expected to be DNA of BBTV. However, 2 banana leaves samples were not detected to be infected by BBTV. These 2 samples were suspected to be infested by other viruses. In addition, the healthy sample was not produced any band on the gel electrophoresis.

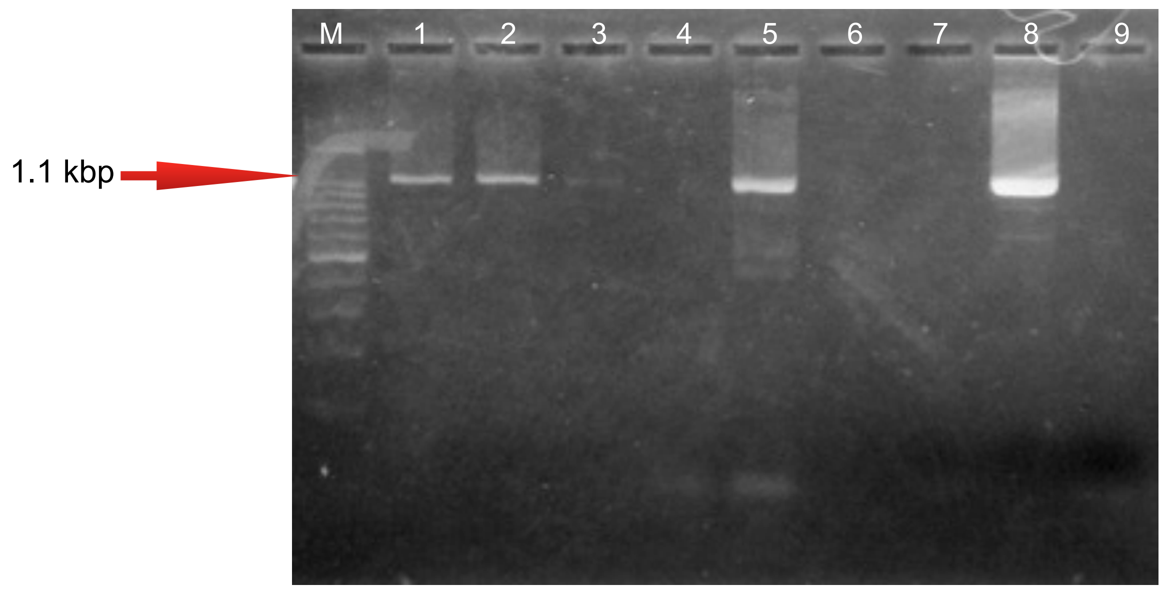

Figure 4: Gel electrophoresis of DNA extracted from banana and abaca leaves. The PCR products were analysed on 2% gel.

No. of well	Samples
M	100 bp ladder
1	BBTV - positive check
2	BBTV - positive banana (Chien)
3	Abaca (Forestry isolate) (Chien)
4	BBTV - positive (Patrick)
5	Abaca cv. Negro (mixed infection) (Patrick)
6	BBTV - positive (Fitri)
7	Banana CES unknown
8	BBTV - positive (Yatie)
9	Banana CES (Yatie)

Based on the result viewed on gel electrophoresis (Figure 4), DNA from 3 samples of infected banana and abaca leaves were successfully extracted [banana (well no. 2, 5 and 8). Amplification of DNA using specific primers of BBTV yielded PCR product with the length fragment approximately 1.1 kbp. These samples showed positive infection of BBTV. All 3 samples were proceed for gel purification and sequencing.

Conclusion

Banana bunchy top virus can be detected using specific BBTV primers. DNA of virus was obtained from fresh and old samples of infected banana using standard DNA extraction protocol. The proper storage of samples are vital in order to ensure the DNA of virus well preserved for future used.

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Appendix 9

Detection of Banana bunchy top virus (BBTV) from Viruliferous Aphids (Direct) and FTA Plant Card

Introduction

Pentalonia nigronervosa is pest of banana. The insect also infest many tropical, subtropical and ornamental plants. Banana aphid was first discovered in Honolulu (1924) (Zimmerman, 1948). The banana aphid have soft body same as mealy bugs, leafhoppers and white flies. There are consist of 2 types; singled (alate) and wingless (apterous). Both of aphids are able to transmit viruses.

Damage

Banana aphid is a phloem feeder, uses its long stylets to pierce plant tissues to suck the sap directly from the vessels. The infected banana will become deformed, the leaves are curled and shrivelled. In some cases, galls are formed on the leaves. Infected young plants can be killed and growth checked if sufficient feeding by banana aphids.

Transmission

The banana aphis transmits in non-persistent manner. The virus was taken up into aphid "mouth" while feeding on the infected plants and transferred to a healthy plant during subsequent feedings. The virus reproduces in the plant and aphids simply aid in transporting the virus. These virus-vector associations shows aphids acquire the virus and it only able to transmit the virus temporarily. In addition, once all the infective charge is reduced by feeding or passing at time, the aphid is unable to transmit the virus until it feeds on infected plant tissue again.

Symptoms

Infected banana will appear as dark green streaking on leaves, midrib and petioles, progressive leaf dwarfing, marginal chlorosis and leaf curling. The diseased fruits is unsaleable because it is small and distorted.

Impact

Recently, infection by banana aphid cause major economic impact due to the Banana bunchy top virus (BBTV) in Asia, Africa and Australia. The disease is endemic throughout South East Asia. Major impact also have been reported on the banana industry in Egypt,

India, Pakistan and Sri Lanka. This aphid also transmits banana mosaic disease in bananas (cause Cucumber mosaic virus - CMV) and cause Abaca bunchy top in *Musa textiles* (abaca/ Manila hemp.).

Materials and methods

a) Direct DNA extraction method from viruliferous aphids (using Nucleon *PhytoPure DNA Extraction Kit, GE Healthcare Life Sciences*)

1. Ten viruliferous aphids were immobilised using 70% ethanol. The aphids was taken from banana infected plant in growth chamber.
2. The ethanol was removed and let the aphids dried.
3. The aphids were grinder in liquid nitrogen. Well grounded sample was mixed with Reagent 1.
4. The sap was transferred into 1.5 mL micro centrifuge tube. The tube was turn upside down or gently shake.
5. After that, 100 uL of DNA extraction Reagent 2 was added and once again turn the tube upside down or gently shake.
6. The samples were vortex for few second and heated for 10 min at 65°C using dry Thermo Unit.
7. Then, the samples were put in cold box for 20 min.
8. 250 uL of chloroform and shake gently.
9. 50 uL of resin was added. The resin was pre-vortex the resin to prevent the sedimentation.
10. The samples were centrifuged at 2500 rpm for 10 min at room temperature. The centrifugation should be repeated if plant debris are not completely settled.
11. First DNA was collected using 125 uL calibration and another 125 uL on the second (total DNA concentration is 250 uL).
12. All samples were added with 250 uL 2-propanol and shake gently.
13. The samples were centrifuged at 15000 rpm for 5 min at room temperature DNA template at the bottom of the tube.
14. 125 uL of supernatant was pipetted out. 100 all of 70% ethanol was added into the pellet.
15. The tubes were centrifuged at 15000 rpm for 2 min at room temperature.
16. The ethanol was pipetted out. The samples were dried out 2 to 3 min at room temperature.

17. 100 uL of 1X TE buffer were added to the samples. The pellet was broke by touching with pipette tip.

b) DNA extraction method from viruliferous aphids impregnated on FTA plant card

1. Ten viruliferous aphids were immobilised using 70% ethanol.
2. The ethanol was removed and let the aphids to dried.
3. Nucleic acid of aphids was impregnated into FTA plant card.
4. One disc was punched from the plant card using Harris puncher, 2.0 mm and was put into 0.2 mL PCR tubes.
5. 200 uL of 90% ethanol was added into the PCR tube and incubated for 5 min at room temperature (RT).
6. The ethanol was removed and another same volume was added again and incubated for 30 min at RT.
7. 200 uL of FTA purification reagent was added and incubated for 5 min at RT.
8. The liquid was decanted and step 5 was repeated two times.
9. 200 uL of 1X TE buffer was added and incubated for 5 min at RT.
10. Step 9 was repeated.
11. The liquid was removed and the disc was dried for 1 - 2 hours. The DNA was ready to be used for PCR amplification.

Figure 1: Samples and DNA extraction reagent. (a) aphids on infected banana; (b) aphids on taro; (c) sampling of aphids from taro into 70% ethanol; (d) aphid in the tube containing 70% ethanol; (e) aphids was dried on tissue; (f) aphids sap after grounded in liquid nitrogen and additional of extraction reagents; (g) reagents used from *Nucleon PhytoPure DNA Extraction Kit.*

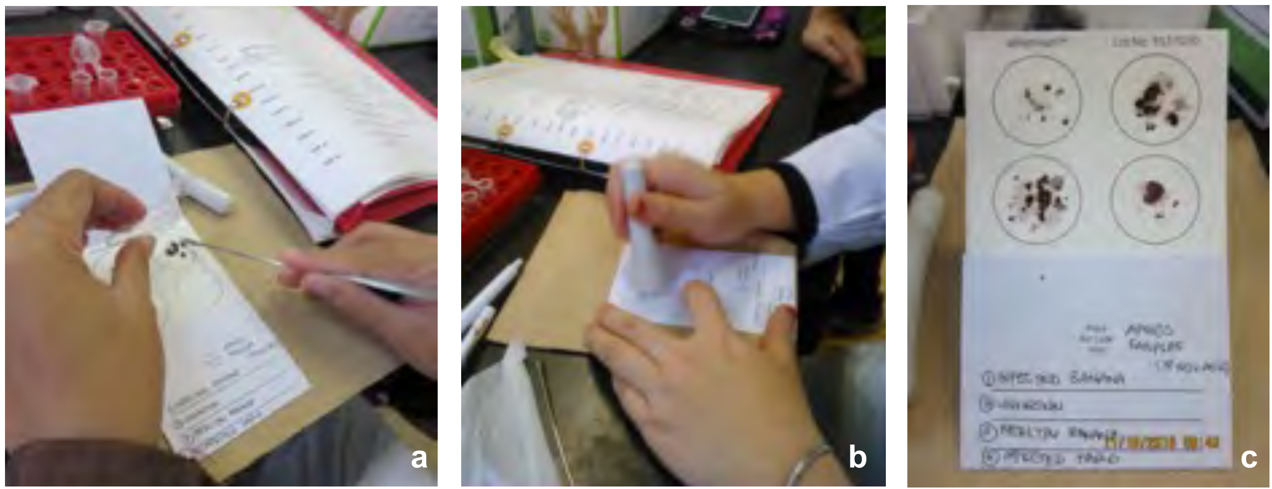

Figure 2: Collection and isolation of DNA from aphids using FTA plant card. (a) placement of aphids; (b) Impregnation of aphids using mortar; (c) the DNA of aphids on FTA plant cards.

Result and discussion

Figure 3: Gel electrophoresis of DNA extracted from banana aphids (direct and FTA plant card). The PCR products were analysed on 2% gel.

No. of well	Samples
M	100 bp ladder
1	BBTV - infected aphids
2	Negative
3	Unknown
4	Taro
5	BBTV - infected aphids

No. of well	Samples
6	Unknown
7	S1 - BBTV - infected aphids
8	S2 - unknown
9	S3 - healthy
10	S4 - taro
11	Taro fresh sample

Based on gel electrophoresis analysis, only 2 samples (genomic DNA was extracted directly from aphids) were positive on Banana bunchy top virus (BBTV) infection. The results were expected to get the bands on the gel. Both samples were severely infested by aphids. The PCR products yielded from both samples was approximately 1.1 kip which identified as BBTV. However, the taro was not infected by BBTV even though the plant in growth chamber observed to be highly infested by the aphids. The taro was suspected to be infested by other viruses (unknown). In order to detect the virus species infected taro plant, Potyviruses primers should be used. The possibility of Taro to be infested by Potyvirus group is high. Potyvirus is a largest group of viruses has been reported to cause diseases on plants.

Conclusion

Both nucleic acid of Banana bunchy top virus (BBTV) from viruliferous aphid can be extracted, collected and isolated using two different methods; by DNA extraction protocol and FTA plant card.

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Detection of Banana bunchy top virus (BBTV) and Banana bract mosaic virus (BBrMV) from FTA Plant Card

Introduction

Detection of plant viruses nowadays become possible and easier with the development of new techniques and technologies upgraded from previous conventional methods. The development of such technologies for example detection of viruses using DNA and RNA, PCR amplification and etc. help researches to discover new viruses and come out with effective solution to prevent the spread of plant diseases. However, researchers still facing some problems on preservation of DNA and RNA especially for long term storage. One of new solution to overcome the difficulty is by FTA card for collection, isolation and storage of nucleic acids.

FTA card provides a safe and reliable technology for room temperature collection, trapped and storage of nucleic acids. The FTA card is suitable for any samples including blood, bacteria, microorganisms, plant materials, viruses and etc by simple application to the card either direct or with an applicator swab. Advantages using FTA card are:

a) Easiest way for DNA collection and isolation: samples are apply directly on the FTA card and allow to dry. The DNA captured and stabilised for immediate processing or long term storage. No heat or centrifuged needed.
b) DNA can be stored at room temperature for several years: genomic DNA stored on FTA card will last long for several years without loosing the PCR efficiency.
c) Stored samples are ready for further analysis less than 30 minutes: nucleic acids captured on FTA card are ready to be used for downstream applications such as PCR, RT-PCR, RFLP and Restriction Enzyme Digestion. Genomic DNA which bound to the punch disc can be repetitively amplified.
d) Safe and secure transportation of DNA: nucleic acids captured on the FTA card is inactive including blood-borne pathogens and prevent the further growth contaminants such as fungus and bacteria.

Therefore, the experiment was conducted to detect BBTV and BBrMV from genomic DNA and RNA collected from banana, abaca and taro leaf samples on FTA plant cards.

Materials and Methods

a) Impregnation of banana samples on FTA card

1. Banana samples was taken from infected plants and cut into small pieces.
2. One small piece of each banana sample was placed onto the FTA card membrane and directly crushed using mortar. The sample should be crushed gently to ensure the DNA was trap into the membrane and avoid it from broken.
3. The samples also can be crushed using mortar and pestle with additional of ionised water (q.s water). The sap then was pipetted onto the membrane.
4. After all the samples were crushed onto the membrane, the card was dried at room temperature for 1 to 2 hours before using for DNA extraction.

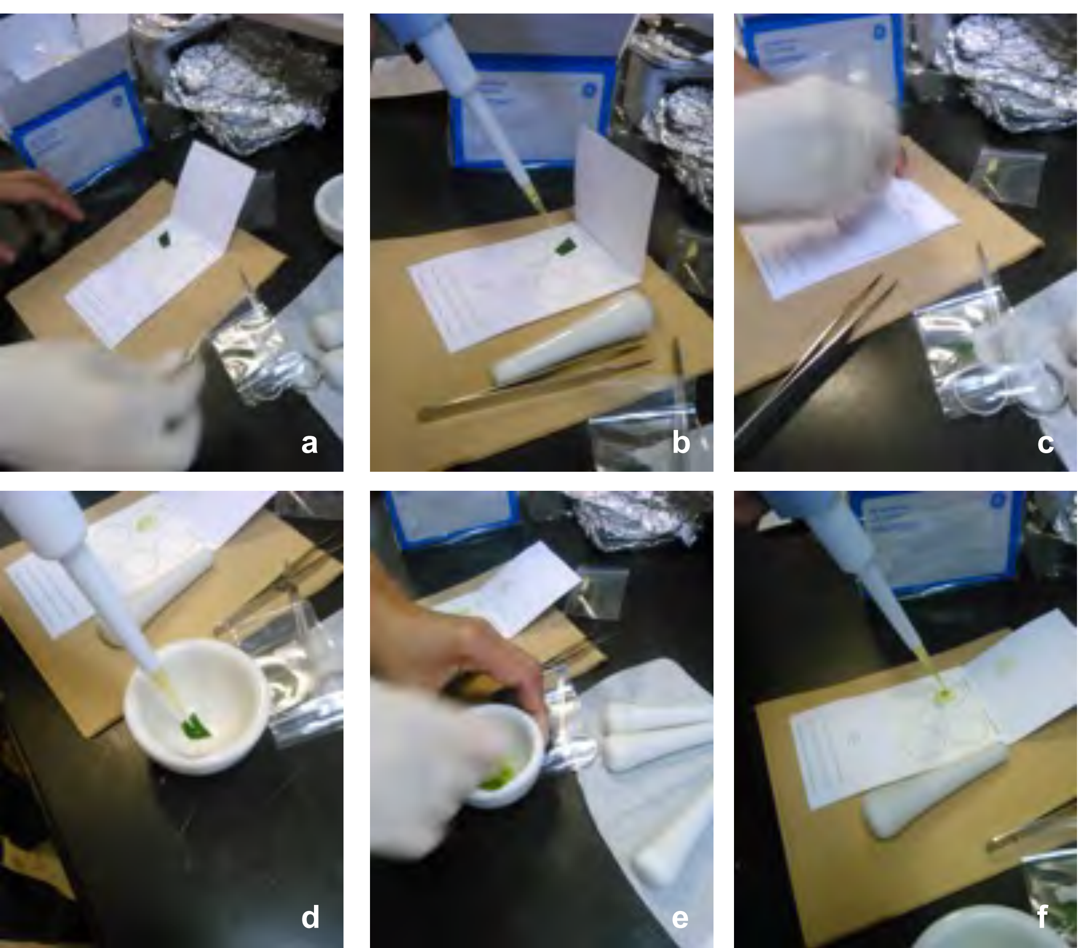
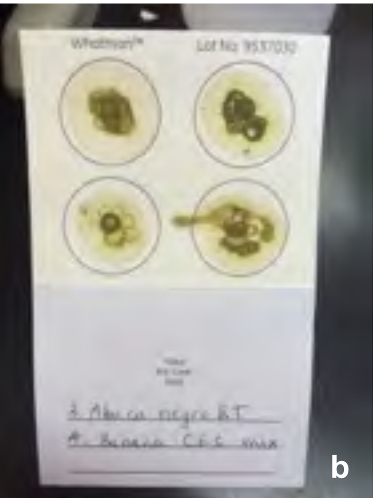
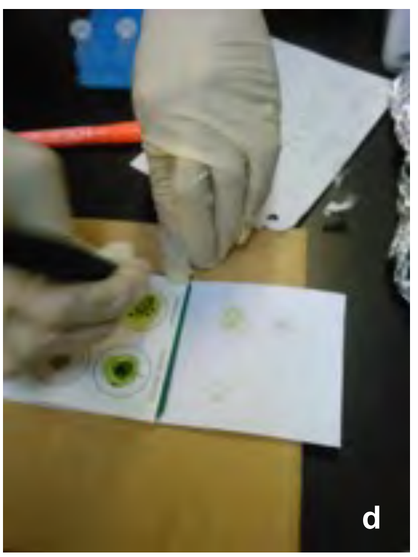


Figure 1: Impregnation samples onto the FTA plant card.
Technique 1 - (a) small size of sample was placed on the membrane; (b) 200 uL of q.s water was put on the sample; (c) sample was crushed using mortar.

Technique 2 - (d) the sample was put inside pestle with addition of water; (e) sample was crushed using mortar and pestle; (f) the sample sap was pipetted on the FTA membrane.





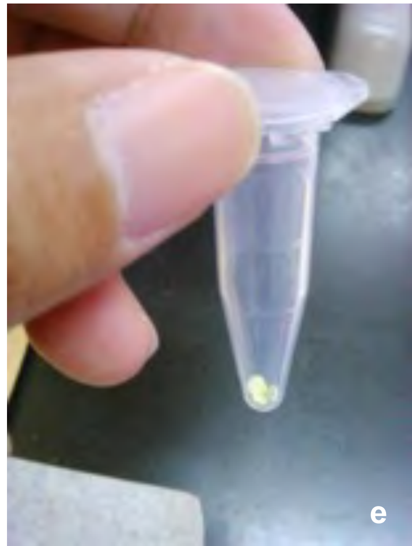


Figure 2: Component of FTA plant card kit and sampling process from FTA plant card. (a) cutting mat and puncher; (b) FTA card with impregnated samples from banana and taro;(c) q.s water (not provided with the kit; (d) Puncher was used to make sample discs; (e) disc was transferred into micro centrifuge tube.

b) DNA extraction from FTA plant card

1. Eight pieces of 2 mm discs was placed into 1.5 ml micro centrifuge tube.
2. 100 uL of processing was added*
3. 1 uL of RNAse inhibitor was added.
4. The samples were incubated on ice for 5 min and mixed every 5 min interval.
5. Supernatant was transferred into new microcentrifuge tube.
6. The supernatant was eluted with 10 uL 3M sodium acetate (pH 5.2).
7. An equal volume (10 uL) of ice-cold 2-propanol was added.
8. The sample then incubated at -80°C for 30 min.
9. Sample was centrifuged at 15000 rpm for 10 min and supernatant was discarded.
10. Pellet (RNA) was washed with 500 uL of 75% ethanol.

11. The pellet was centrifuged again at 15000 rpm for 2 min and air dried for 2 min.
12. The sample was re-suspend in DEPC-treated water (30 uL).
13. The sample was used for cDNA synthesis following REVERT protocol and proceed with PCR using TAKARA kit.

*Processing buffer for FTA plant card (per 200 mL stock solution)

Final concentration	Stock solution	
10 mM Tris-HCL, pH 8.0	1 M Tris-HCL, pH 8.0	= 2 mL
0.1 mM EDTA	0.5 M EDTA	= 40 uL
200 - 250 ug/mL glycogen		= 0.04 g or 40 mg
2 mM DTT	1 M DTT	= 0.4 mL
distilled water		= 200 mL ++

800 U/mL RNAse out (to be added separately in tube)

c) Synthesis of cDNA (Reverse Transcriptase: RT)

Material	For production of cDNA (uL)
5X RT buffer	4.0
dNTP mixture (10 mM)	2.0
Primer (10 pmol/uL) [oligo(dT) or specific reverse primer]	1.0
RNAse inhibitor (10U/uL)	1.0
ReverTra Ace^{TM} [put in the ice box]	1.0
Total RNA	11.0
Total (uL)	20

The PCR mixture was vortex and used for cDNA synthesis.

Result and Discussions

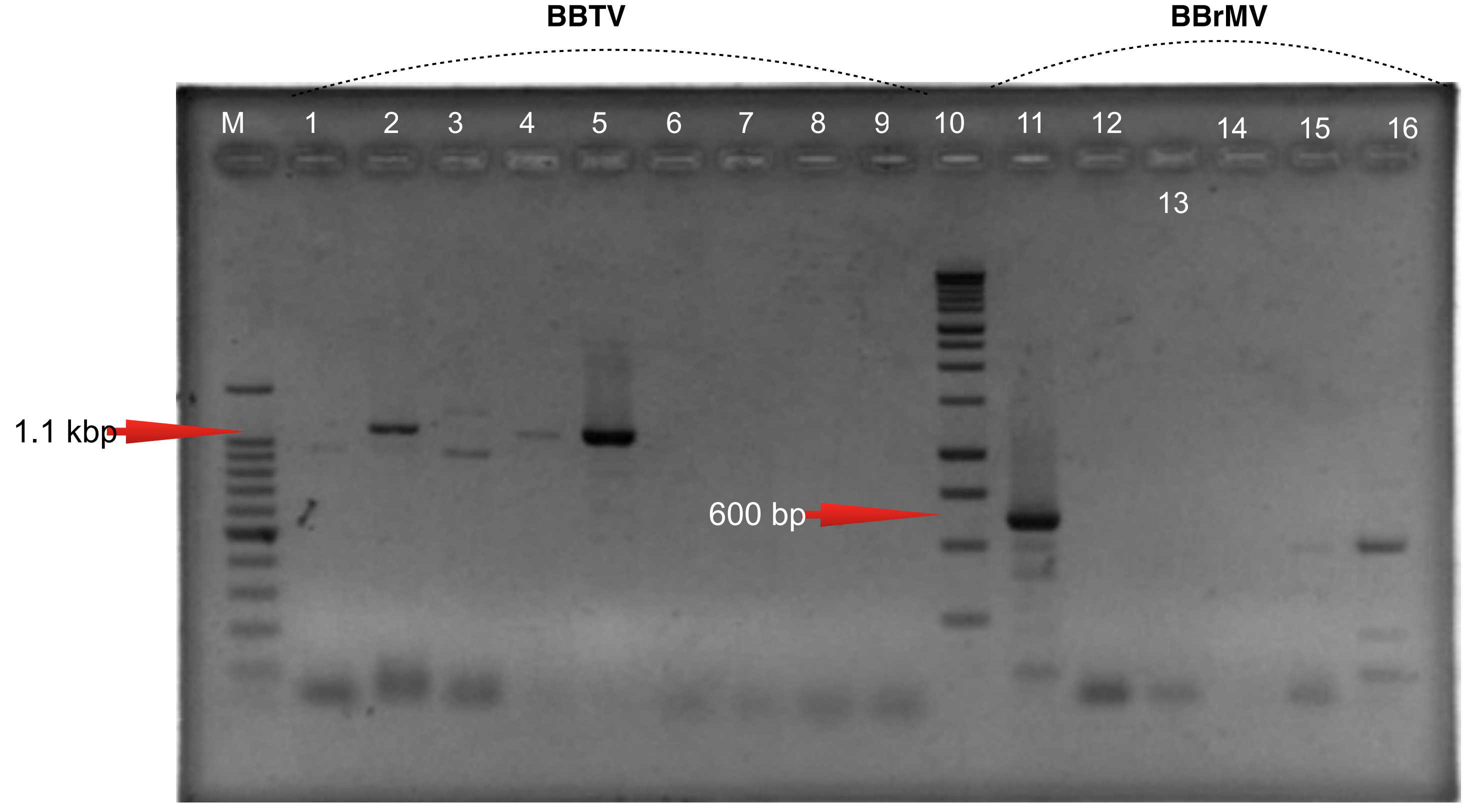


Figure 3: Gel electrophoresis of DNA extracted from banana, abaca and taro leaves for detection of banana bunchy top virus (BBTV) and Banana bract mosaic virus (BBrMV). The PCR products were analysed on 2% gel. Samples were extracted from FTA plant card.

No. of well	Samples
M	100 bp ladder
1	S1 - BBTV - positive banana
2	S2 - BBTV - Abaca Forestry sample
3	S4 - Banana CES mixed infection
4	NODAI sample BBTV - positive (direct impregnation)
5	NODAI sample BBTV - positive (with q.s)
6	Banana CES (non FTA: repeat)
7	Banana BBTV - positive (non FTA: repeat)
8	S2 - unknown
9	S4 - unknown
10	1 kb ladder
11	S3 - Abaca
12	S2 - Abaca Forestry isolate

No. of well	Samples
13	S4 - Banana CES mixed infection
14	Taro
15	NODAI sample BBTV - positive (direct impregnation)
16	NODAI sample BBTV - postive (with q.s)

Based on result obtained from gel electrophoresis, sample from Abaca Forestry and both from NODAI (direct impregnation and mix with q.s) showed positive on BBTV infection. The expected bands for positive BBTV were approximately 1.1 kbp. However, negative BBTV was showed by banana CES and banana positive BBTV, respectively. These two samples were extracted using direct DNA extraction protocol. Non specific bands were obtained from sample S1 and S4. For Banana bract mosaic virus (BBrMV), only one sample (Abaca) was showed positive infection with the DNA length is approximately 600 bp. There are negative BBrMV infection from Abaca Forestry isolate, banana CES mixed infection and taro. Two samples were showed non specific bands.

The non specific binding of PCR product are due to the several reasons:

a) Contaminants in primers may inhibit the PCR.
b) Impure of dNTPs were used. Contaminants in the dNTPs mix can lead to incomplete or incorrect amplification or PCR inhibition.
c) Impure water was used. Water would have been contaminated during prior pipetting errors.

To overcome these problems, these precaution should be taken during PCR mixture preparation:

i) Use desalted primers or more high purified primers.
ii) Dilute the primers to determine if inhibitory effects exist but do not less than 0.02 uM for each primers.
iii) Use high quality of dNTPs.
iv) Use fresh nuclease free water.

Conclusion

FTA plant card is one of the best option to collect and preserve DNA of viruses. Both genomic DNA and RNA can be obtained from virus infection samples. Banana bunchy top virus (BBTV) and Banana bract mosaic virus (BBrMV) can be detected using PCR amplification from both DNA and RNA. Detection of BBrMV was done by synthesis of cDNA through Reverse Transcriptase PCR (RT-PCR) from RNA obtained from extraction of FTA plant card.

References

1. PCR troubleshooting (Retrieved from: http://www.bio-rad.com)
2. FTA card (Retrieved from: http://www.parliament.vic.gov.au)

Appendix 11

Sample Preparation for Sequencing

Materials and methods

a) PCR Protocol

Materials	For sequencing (uL)
q.s.	34.8
10X *Ex Taq* Buffer	5
dNTP mixture	4
Forward primer (usu. 25 pmol)	0.5
Reverse primer (usu. 25 pmol)	0.5
TaKaRa Ex Taq (5 units/uL)	0.2
Template cDNA	5
Total (uL)	50

Procedures:

1. Cocktail mix was prepared and required amount was calculated (follow the sequence in adding chemicals as shown in the table).
2. Cocktail mix with 45.25 uL was dispensed into PCR tube.
3. DNA extract, 2.5 uL was added. PCR tubes were flashed for a few second to eliminate any bubbles.
4. The PCR machine was ran. After the cycles finished, samples were viewed by gel electrophoresis.
5. Precaution: *TaKaRa Ex Taq* should be placed in cold box. Do not touch the bottom of the tube with hands since it is temperature sensitive. Extra precaution should be taken in staining and de-staining the gel in ethidium bromide (EtBr). Gloves must be used.

b) Gel Electrophoresis

1. 2% gel was prepared.
2. Blue juice/loading dye was added, 2 uL on parafilm (droplets).
3. For 8 band-comb (small comb), 8 uL of PCR product used on 2 uL blue juice/loading dye.
4. The samples were loaded into gel starting with 100 bp ladder/marker (for BBTV) at 13 uL; followed by the negative sample.

5. The gel was loaded, the orientation should be from negative (-) to positive (+) at 100 V for 25 - 30 min.
6. The gel was stained in EtBr for 5 min.
7. Then, the gel was de-stained with distilled water.
8. Finally, the DNA bands were viewed under UV illumination and photo was taken using EDAS 290 (Kodak, Japan).

	Big gel
Agarose powder (g)	0.5
1X TAE Buffer (mL)	25

c) Gel cutting and purification

1. After the gel electrophoresis, the DNA bands were viewed under UV transilluminator.
2. Empty 1.5 mL micro centrifuge tube was weigh.
3. The DNA band was sliced with scalpel and transferred into the 1.5 mL micro centrifuge tube.
4. The gel slice was weigh and 3 volumes of QG buffer was added to 1 weigh go gel.

Calculation of QG buffer.

i) weigh of tube = 0.91 g

ii) weigh of tube + gel = 1.08 g

iii) weigh of gel = (ii) - (i) = 0.17 g

Therefore, the volume of QG buffer:

$$\frac{10\ \text{uL}}{0.01\ \text{g}} = \frac{x\ \text{uL solution}}{\text{weigh of gel}}$$

$$= 170\ \text{uL}$$

d) Gel extraction (DNA purification by centrifugation)

I. Dissolving the gel

1. 10 uL of membrane binding solution was added per mg of gel slice.
2. The sample was vortex and incubated at 50 - 65°C for 10 - 15 min or until the gel slice is completely dissolved.

II. Binding DNA

1. SV Minicolumn was inserted into collection tube.
2. Dissolved gel mixture or prepared PCR product was transferred to the mini column assembly.
3. The mixture was incubated at room temperature for 1 min and centrifuge at 16000 rpm for 1 min.
4. Flowthrough was discarded and reinsert mini column into collection tube.

III. Washing

1. 700 uL membrane wash solution (ethanol added) was added.
2. The tube was centrifuge at 16000 rpm for 1 min.
3. Flowthrough was discarded and reinsert the mini column into collection tube.
4. Step 1 was repeated with 500 uL membrane wash solution.
5. The tube was centrifuge at 16000 rpm for 5 min.
6. The collection tube was empty and recentrifuge the column assay for 1 min with micro centrifuge lid open to allow evaporation of any residual ethanol.

IV. Elution

1. Minicolumn was carefully transferred to clean 1.5 micro centrifuge tube.
2. 30 uL nuclease free water was added to the mini column.
3. The tube was incubated at room temperature for 1 min.
4. The tube was centrifuge at 16000 for 1 min.
5. Minicolumn was discarded and DNA stored at 4°C or 20°C.

e) Ligation (to prepare the recombinant plasmid)

1. All reagent and PCR product was mixed with micropipette and incubated overnight at 4°C. Longer incubation time will increase the number of transforming. Generally, incubation overnight at 4°C will produce the maximum number of transformants.
2. 2X Rapid ligation buffer should be vortex prior to use. The 2X Rapid ligation buffer contains ATP which degrades during temperature fluctuations. Multiple freeze-thaw cycles should be avoided and exposed to frequent temperature changes by taking single-use aliquots of the buffer.

f) Transformation

1. Competent *E. coli* (105 uL/tube) was leave in ice bath for 1 - 1.5 hours. Gently mixed it since competent *E. coli* is extremely fragile.
2. 50 uL of competent *E. coli* was added to ligated DNA (recombinant plasmid) and incubated in ice bath for 30 min.
3. The sample was placed in heat block at 42°C for 45 seconds and immediately transfer to ice bath for 2 min.
4. Aseptic technique was done under the laminar flow, 1000 uL S.O.C. medium was added into competent *E. coli* and the tube was wrapped with parafilm.
5. The tube was shaken (horizontally) at 150 rpm for 45 min at 37°C.
6. After shaken, the tube was centrifuge for 2 min to get pellet of *E. coli*.
7. The S.O.C. medium was poured off leaving about 200 uL of the medium in the tube.
8. The tube was vortex for about 15 sec.
9. In the laminar flow, the mixture was dropped onto LB medium and plate was spreader with sterile triangle rod until completely spread and dried. The plate was wrapped with parafilm.
10. The plate was incubated at 37°C overnight and transferred to 4°C for 1 - 2 days. The plate was leave upside down.

g) Culturing *E. coli* in TB medium

1. Five white colonies of *E. coli* were selected from the culture.
2. Using the sterile toothpick, a single colony was transferred into TB medium.
3. One blue colony was included as negative control.
4. The used toothpick was flamed afterwards.
5. The tube was wrapped with parafilm and incubated at 37°C and shaken overnight.

h) Mini Prep

Plasmid DNA was isolated and purified using LaboPass^{TM} Plasmid Mini Kit (*Hokkaido System Science, Co. Ltd. Japan and DNA Purification Kit, COSMO GENETECH*)

1. After shaking overnight, the tube was centrifuge at 3500 rpm (TOMY LC-100 Low speed centrifuge) for 7 min. The E. coli pellet was observed.
2. TB medium was poured off into separate flask and the pellet was kept (pellet size about 1 cm)

3. 250 uL buffer S1 was added (the tip should not touch the tube). In S1 buffer, RNAse must be added prior to use.
4. The pellet of E. coli was re-suspend and vortex for about 25 - 30 sec.
5. All suspension was transferred into 1.5 mL tube.
6. 250 uL buffer S2 was added (buffer S2 must be shaken before use) and then the tubes were inverted 3 - 4 times (do not vortex). Inverting the tubes will totally mix the cells with the buffer.
7. The tubes were incubated at room temperature for 1 - 5 min.
8. 350 uL buffer S3 (buffer S3 must be shaken before use) and 2 phases was appeared - clear and aqueous phase. It shows that the cells are already disrupted.
9. The tube were inverted gently 3 - 4 times.
10. The tubes were centrifuge for 10 min at 14000 rpm and E. coli was appeared at one side of the tubes.
11. Th DNA plasmid (clear solution) into the spin column. The plasmid has been trapped in the filter.
12. The tubes were centrifuge for 1 min at 14000 rpm.
13. The liquid poured off, 750 uL of buffer PW was added and centrifuge for 1 min at 14000 rpm. (PW buffer will further wash the filter to purify the plasmid).
14. The liquid was poured off, centrifuged for 1 min at 14000 rpm to completely dry the filter.
15. The spin column was removed and transferred it into 1.5 mL tube.
16. 50 uL of buffer EB was added into the centre of the filter and left at room temperature for 1 min. (The buffer EB will dissolved the plasmid from the filter).
17. The tube was centrifuge for 1 min and kept the DNA plasmid. (Plasmid is in the 1.5 mL tube and then the spin column was discarded).

i) Insert Check

1. 1 - 1.5% of agarose gel prepared.
2. 2 uL of loading buffer was mixed (6X load dye) and 2 uL negative control. The negative control is the blue colony.
3. 2 uL loading buffer and 2 uL sample was mixed separately.
4. Each sample was loaded onto the gel.
5. The gel was ran for 30 min.
6. The gel then was stained in EtBr for 3 min and rinsed with distilled water.
7. The bands were viewed under UV transluminator.

j) Cycle Sequence

Materials	For sequencing (uL)
q.s. (double distilled water)	2.0
Sequence buffer (Big dye Terminator 5X sequencing buffer	1.0
Primer (sp6/T7)	0.5
Premix	2.0
DNA (PCR product)	4.5
Total (uL)	10.0

Subject to Thermal Cycle: Sequence Program

1 - 24 cycle	step 1	96°C	10 sec
	step 2	50°C	5 sec
	step 3	60°C	4 min
25 cycle		4°C	forever

(about 3 hours)

k) Precipitation

1. After thermal cycle, 1.5 mL tube was prepared.
2. The sample (cycle sequencing product) used = 10 uL
   3M acetic acid = 1 uL
   99.5 - 100% EtOH = 30 uL

Optional step: The sample can be kept at -30°C for a long time or proceed to the next sequencing procedure.

3. The tube was mixed and centrifuge briefly.
4. The sample was put in ice block for 10 min.
5. The sample was centrifuge at 20°C about 20 min at 14000 rpm.
6. The supernatant was discarded and the pellet was kept.
7. 100 uL of 99.5% EtOH (not cold) was added and centrifuge for 5 min at 14000 rpm.
8. Step 6 was repeated.
9. 100 uL of 99.5% EtOH was added and rolled horizontally about 20 sec.
10. The tube was centrifuge for 5 min at 14000 rpm.
11. The supernatant was discarded and pellet was dried for 2 min at room temperature (open lid).
12. The tube was put in heat block at 95°C for 2 min (open lid).

13. Then, the tube was put in ice block for 5 min (close lid).
14. The pellet was kept at 4°C.

I) DNA analysis using Automate Sequencer (*Applied Biosystem 3130xl Genetic Analyser, HITACHI*)

1. The pellet was dissolved with 20 uL Hi-Di Formamide.
2. The sample was put into the plate carefully (should have bubbles).
3. The plate was put into the machine.

Sequencer machine

a. The computer and machine was turned on.
b. Program 3130xl data collection v30 was opened.
c. Program check (red to yellow to green light).
d. 1X Buffer EDTA was changed and used about 16 mL.
e. Polymer 3130 POP 7 was changed.
f. 1X buffer in the bottle was changed.
g. Bubble was discarded.
h. The program was ran and waited until the temperature goes to 60°C.

Note: a map must prepared based on the sample arrangement in the plate. 1X buffer EDTA was prepared by adding 3600 uL 10X EDTA buffer to 36 mL distilled water. All buffers were changed after using it for 1 week and washed and wiped with water. 3 containers are water and 1 with 1X EDTA buffer.

Figure 1: (a) and (b) Gel cutting under UV light; (c) Promega Gel Purification Kit. The gel was then proceed for gel purification protocol.

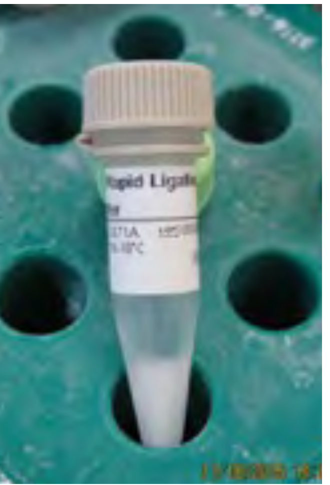


Figure 2: Reagents using for ligation to prepare recombinant plasmid.

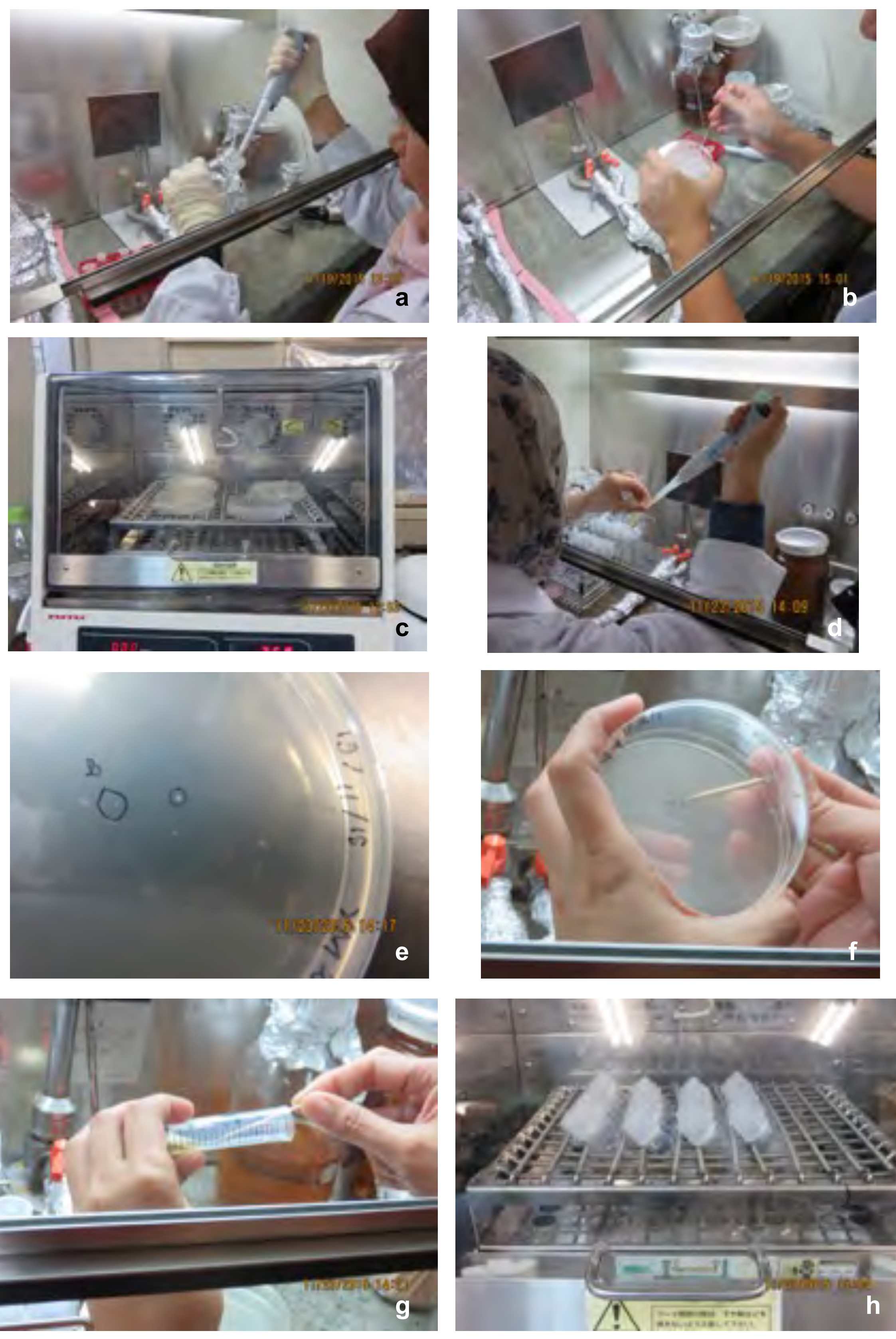


Figure 3: (a) Dispensing of S.O.C medium for production of competent *E. coli*; (b) Culturing competent *E. coli* on LB medium; (c) Incubation of *E. coli* plate in the incubator; (d) Dispensing of TB medium for culturing of E. coli; (e) Blue (without insert) and white (with insert) *E. coli* colony on LB medium; (f) Picking up *E. coli* colony using toothpick; (g) Transferring *E. coli* colony into TB medium; (h) Incubation of *E. coli* (with and without insert in the incubator shaker.

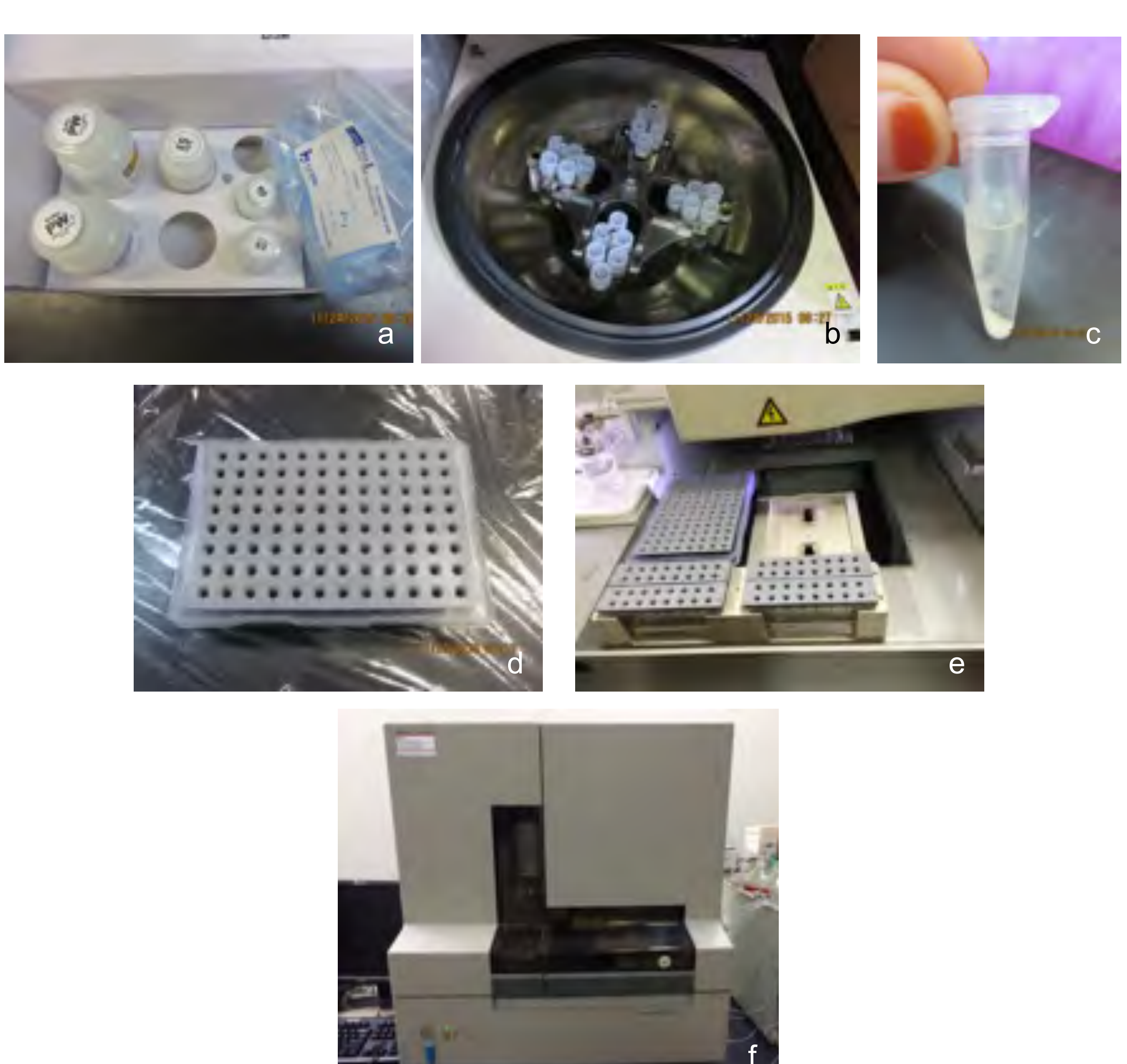


Figure 4: (a) Plasmid Mini Kit for plasmid DNA purification; (b) Plasmid DNA in centrifuge; (c) Precipitation of DNA; (d) Plate using for DNA sequencing; (e) Plate was placed in the sequencer machine; (f) Automate sequencer for DNA analysis.

Results and Discussion

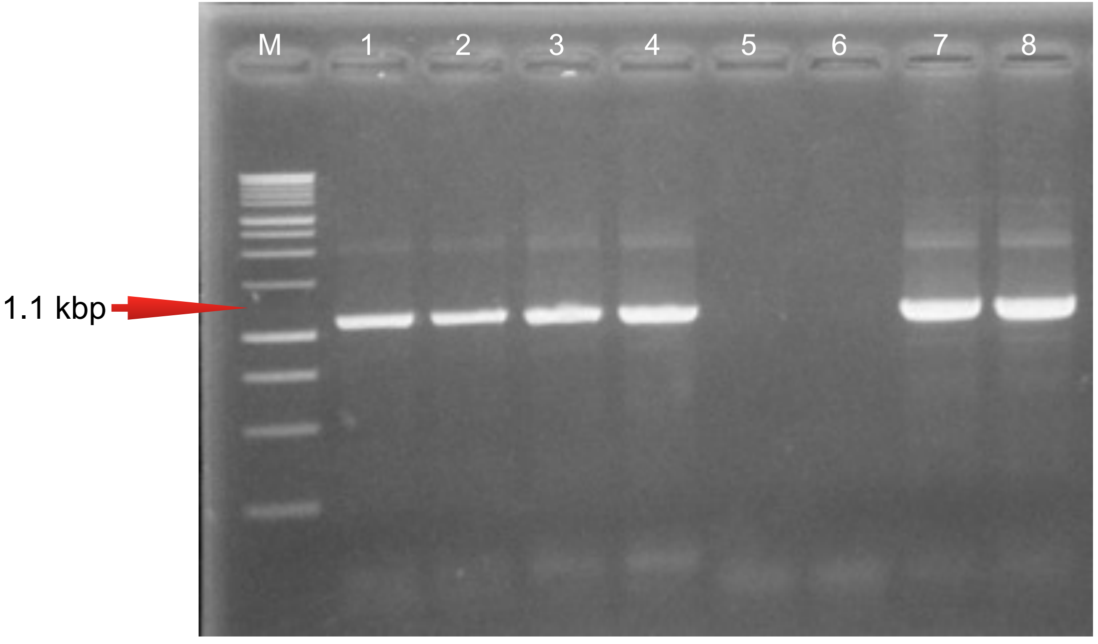


Figure 5: Gel electrophoresis of DNA extracted from banana for gel cutting. The PCR products were analysed on 2% gel.

No. of well	Samples
M	1 kbp ladder
1	BBTV - positive (old sample) Chien
2	BBTV - positive (old sample) Chien
3	Abaca cv. Negro (old sample) Patrick
4	Abaca cv. Negro (old sample) Patrick
5	BBTV - NODAI sample Fitri
6	BBTV - NODAI sample Fitri
7	Banana CES (old sample) Yatie
8	Banana CES (old sample) Yatie

Based on the gel electrophoresis in Figure 5, 3 samples of BBTV was chosen for the gel cutting. The DNA bands were approximately 1.1 kbp in length which is identified as Banana bunchy top virus (BBTV).

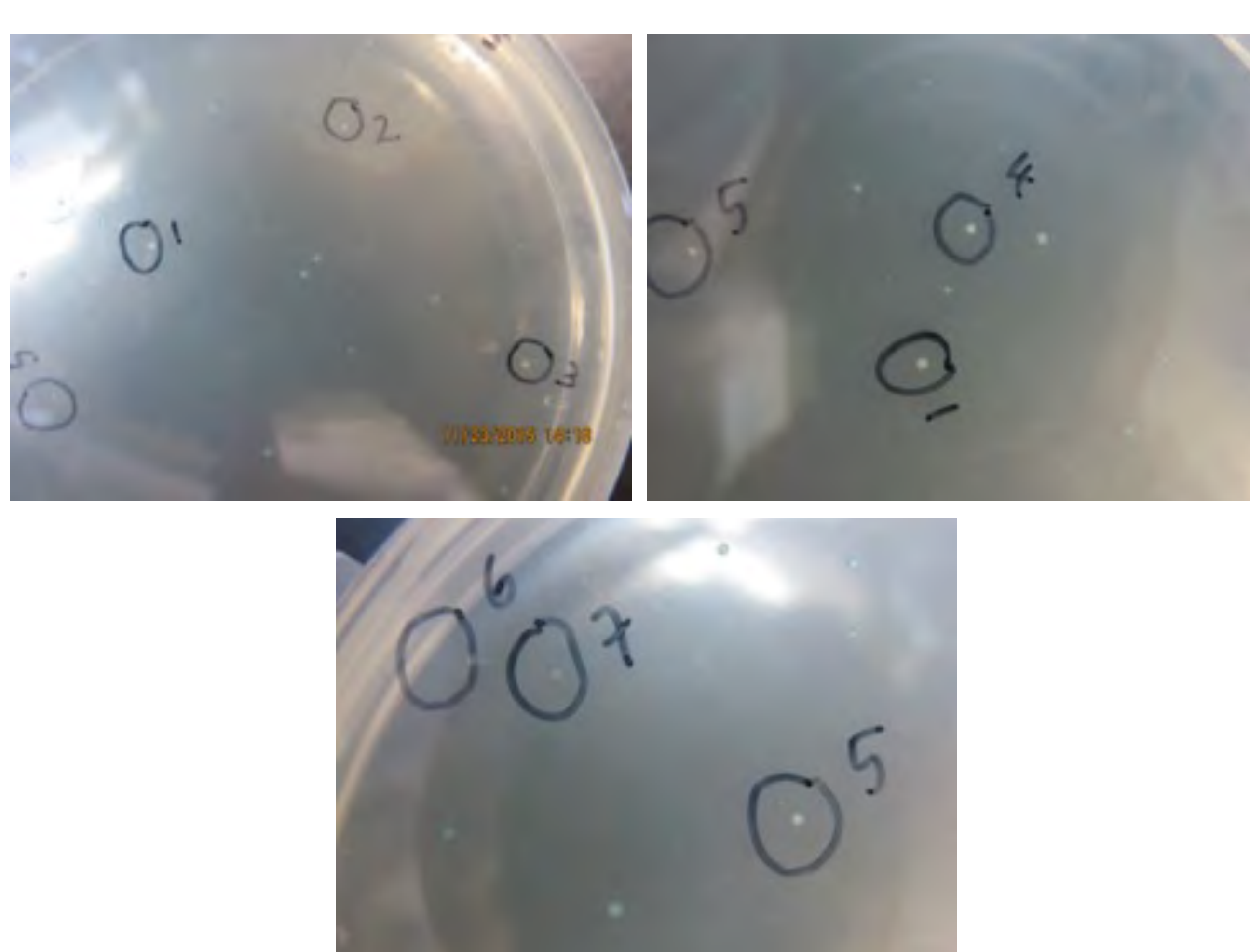


Figure 6: Colony of *E. coli* growth on TB media and was chosen for insert check procedure. White colony labelled with No. 1 - 7 are white colony which indicate to have virus insert. Blue colony (not labelled) have no virus insert.

E. coli colony was growth on the TB media for more than 24 hours. The plate was maintained in the 4°C refrigerator for 2 days to prevent overgrowth of the bacterial cell. Before insert check procedure was done, the colony should be observed carefully to make sure only white colony should be chosen. Plasmid DNA of E. coli was purified first before undergo insert check procedure.

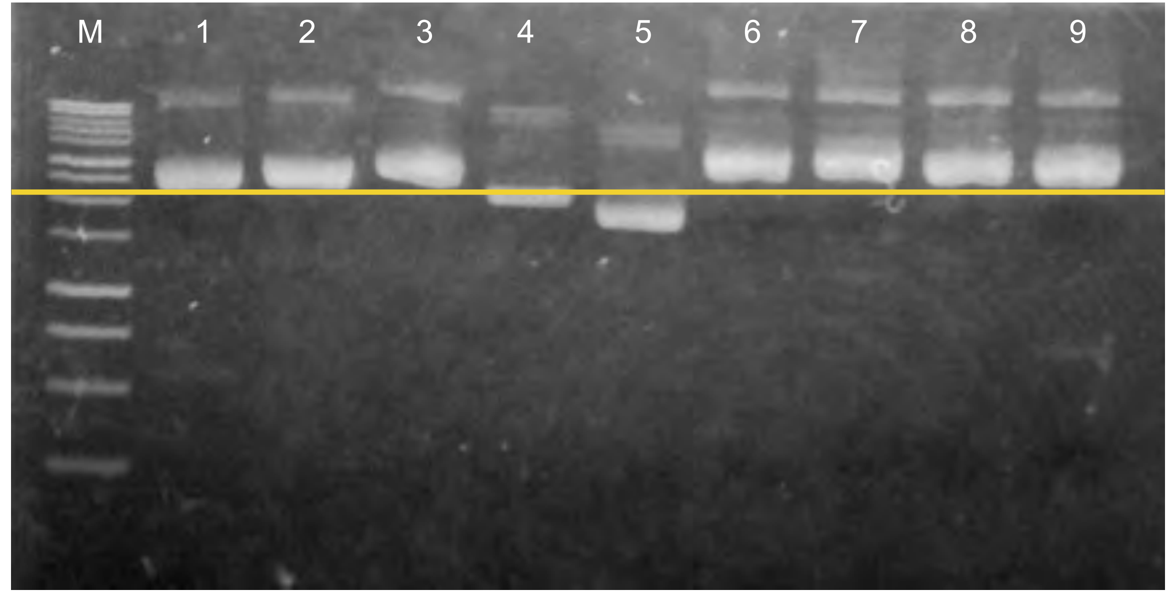


Figure 7: Insert check for plasmid DNA by 1.5% gel electrophoresis. The yellow line indicates the DNA length is 4.1 kbp (3000 bp).

No. of well	Samples
M	1 kbp ladder
1	Negative Insert (blue colony)
2	Positive Insert - NODAI sample Fitri - white colony
3	Positive Insert - NODAI sample Fitri - white colony
4	Positive Insert - NODAI sample Fitri - white colony
5	Positive Insert - Banana CES Yatie - white colony
6	Positive Insert - Banana CES Yatie - white colony
7	Positive Insert - Banana CES Yatie - white colony
8	Positive Insert - Banana CES Yatie - white colony
9	Positive Insert - Banana CES Yatie - white colony

Based on insert check result (Figure 7), plasmid DNA in well No. 2 to No. 5 was eliminated for cycle sequence. Only 4 samples of plasmid DNA indicate the successful ligation and were chosen (the DNA length is approximately more than 4.1 kbp) for cycle sequence step. These for 4 bands were showed to have heavy weight compared to the other unsuccessful plasmid and negative insert. There are several reasons for unsuccessful insert during insert check;

a) Failure during transformation step - The *E. coli* was not incubated properly in 4°C to produce transformant.
b) Nucleic acid of virus is not successfully inserted into the vector.
c) Mistake in picking the white colony during culturing the *E. coli* in TB medium. Picking up the blue colony will appear in gel electrophoresis (the length of DNA is less than 4.1 kbp).

Therefore, only samples No. 6, 7, 8 and 9 for sequencing process to identify the viruses.

Sequencing results

Consensus sequence obtained from 4 clones:

GGCGGAGCCATGTCATCGAAGGGATTATTTGGGTCTACGGACCAAATGGAGGCGAAG
GAAAGACAACGTTTGCAAAACATTTAATGAAGACTAAGAATGCGTTTTATTCGCCAGGA
GGAAAATCATTGGATATATGTAGATTGTATAATTATGAGGATATAGTTATATTTGATATTCC
CAGATGCAAAGAGGAATATTTAAACTATGGTTTATTAGAAGAATTTAAAAATGGAATTATT
CAAAGCGGGAAATATGAACCCGTTTTGAAAATTGTAGAATANGTGGAAGTCATTGTAAG
GCTAACTTCCTTNCNANGGAAGGAATCTTTTCTGAAGATCGAATAAAGCTAGTTGCTTG
CTGAACACGCTATGACAATCGTACGCTATGACAAAAGGGGAAAAGCAAAGATTCGGGG
GTTGATTGTGCTATCCTAACGATTAAGGGCCGCAGGCCCNTNANGATGGACGACGCG
ATCATATGTCCCGAGTTAGTGCGCCACGTAAGCGCTGGGGCTTATTATTACCCCCAGC
GCTCGGGACGGGACATTTGCATCTATAAATAGACCTCCCCCCCCTCCACTACAAGATC
ATCATCGTCGACAGAAATGGCGCGATATGTGGTATGCTGGATGTTCACCATCAACAATC
CCGCTTCGCTACCAGTGATGCGGGATGAGTTTAAATATATGGTATATCAAGTGGAGAGG
GGACAGGAGGGTACTCGTCATGTGCAAGGATACGTCGAGATGAAGAGACGAAGCTCT
CGTAAGCAGATGAGAGGCTTCTTCC

This consensus sequence was used in NCBI website to identify the virus. From blast result, the virus was identified as Banana Bunchy top virus (BBTV) which shows 100% similarity to Banana bunchy top virus isolate Kerala 2 segment DNA-1 complete sequence.

Conclusion

Molecular technique is a very useful tool in identification of plant pathogens specifically plant viruses. Plant viruses cannot be identify only based on symptoms observed in the field because misidentification of diseases will be occur. ELISA method can be one of the option, however the availability of antibody can be the main constraint to identify those pathogens. Therefore, researchers are prefer to used this method to get fast, accurate and reliable results for virus identification.

Appendix 12

RNA Extraction of Potyviruses from Passion Fruit using Phenol Chloroform

Materials and Method

a) RNA Extraction

*Glove must be wore. Aluminium foil should be spreader on the lab bench and sterilised with ethanol.

1. Freeze mortar and pestle should be used for the extraction protocol. The centrifuge temperature was set at 4°C.
2. Buffer for the extraction was prepared using RNase free Eppendorf tube according the following ratio:

2X STE	1 mL
SDS	0.01 g
2-mercaptoethanol	10 uL

*Vortex both 2X STE and SDS before mixed with 2-mercaptoethanol.

3. Six 1.5 mL of RNase free tubes were prepared per sample.
4. 0.1 g of leaf was put in mortar and liquid nitrogen was added and refrigerated the sap for a few moments.
5. If the leaf is shattered, 800 uL buffer was added for extraction.
6. 1st centrifugation of sample - 5 min at 15000 rpm. The top layer of the sample (**600 mL**) was transferred into new RNase free tube. Same amount of phenol chloroform was added into the top liquid.
7. 2nd centrifugation - 5 min, at 15000 rpm. **500 uL** of top layer of the sample was transferred into new RNase free tube. Same amount of phenol chloroform was added into the top liquid.
8. 3rd centrifugation - 5 min, at 15000 rpm. **400 uL** of top layer of the sample was transferred into new RNase free tube. Same amount of phenol chloroform was added into the top liquid.
9. 4th centrifugation - 5 min, at 15000 rpm. **300 uL** of top layer of the sample was transferred into new RNase free tube. Same amount of phenol chloroform was added into the top liquid.
10. 5th centrifugation - 5 min, at 15000 rpm. **150 uL** of top layer of the sample was transferred into new RNase free tube. Same amount of phenol chloroform was added into the top liquid.

11. 1/3 amount of lithium chloride was added into the top liquid and pipetted slowly. The tube was incubated in the ice for 1 hour.
12. After incubation, the tube was centrifuged at 15000 rpm for 15 min. Then, set the temperature of heat block at 65°C.
13. The supernatant was discarded and left the outside pellet inside the tube.
14. 150 uL of 70% of ethanol was added into the pellet and centrifuged again at 15000 rpm for 5 min.
15. The supernatant was discarded and left the outside pellet inside the tube.
16. 150 uL of 100% of ethanol was added into the pellet and centrifuged again at 15000 rpm for 5 min.
17. The supernatant was discarded and left the outside pellet inside the tube. The tube was dried in upside down position for 10 min.
18. The pellet was dissolved with 110 uL DEPC.
19. The tube was heated shock for 10 min and transferred into the ice box for another 10 min.
20. The RNA extraction product was stored in -20°C.

b) Synthesis of cDNA of potyviruses

Material	For production of cDNA (uL)
5X RT buffer	4.0
dNTP mixture (10 mM)	2.0
Primer (10 pmol/uL) [oligo(dT) or specific reverse primer]	1.0
RNAse inhibitor (10U/uL)	1.0
ReverTra Ace^{TM} [put in the ice box]	1.0
Total RNA	11.0
Total (uL)	20

c) PCR Protocol

Materials	**For detection (uL)**
q.s.	17.4
10X *Ex Taq* Buffer	2.5
dNTP mixture	2
Forward primer	0.25
Reverse primer	0.25
TaKaRa Ex Taq (5 units/uL)	0.1
Template cDNA	2.5
Total (uL)	25

Procedures:

1. Cocktail mix was prepared and required amount was calculated (follow the sequence in adding chemicals as shown in the table).
2. Cocktail mix with 22.5 uL was dispensed into PCR tube.
3. DNA extract, 2.5 uL was added. PCR tubes were flashed for a few second to eliminate any bubbles.
4. The PCR machine was ran. After the cycles finished, samples were viewed by gel electrophoresis.
5. Precaution: *TaKaRa Ex Taq* should be placed in cold box. Do not touch the bottom of the tube with hands since it is temperature sensitive. Extra precaution should be taken in staining and de-staining the gel in ethidium bromide (EtBr). Gloves must be used.

d) Gel Electrophoresis

1. 1.5% gel was prepared.
2. Blue juice/loading dye was added, 2 uL on parafilm (droplets).
3. For 6 band-comb, 13 uL of PCR product was mixed into blue juice/loading dye by repeated pipetting.
4. The samples were loaded into gel starting with 1 kbp ladder/marker at 6 uL; followed by the negative sample.

5. The gel was loaded, the orientation should be from negative (-) to positive (+) at 100 V for 25 - 30 min.
6. The gel was stained in EtBr for 3 min.
7. Then, the gel was de-stained with distilled water.
8. Finally, the DNA bands were viewed under UV illumination and photo was taken using EDAS 290 (Kodak, Japan).

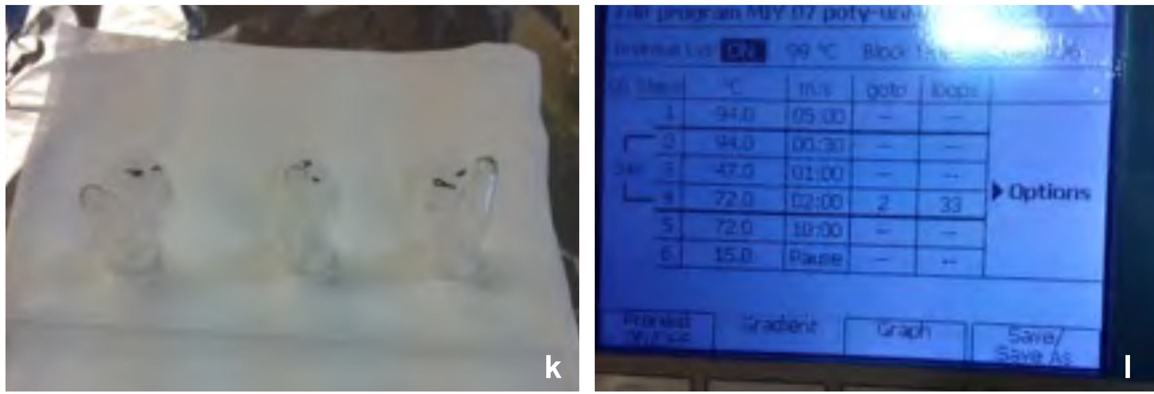


Figure 1: Procedure of RNA extraction. (a) materials needed for RNA extraction; (b) passion fruit infected a month ago with potyvirus; (c) leaf sample was added with liquid nitrogen; (d) sample was mixed with RNA extraction buffer; (e) the sample after additional of phenol chloroform and centrifuged; (f) the top layer (yellow) was transferred into the new RNase free tube; (g) additional of phenol chloroform for 2nd centrifugation; (h) the supernatant was mixed with lithium chloride; (i) sample was mixed with 70% ethanol; (j) additional of 99.5% ethanol; (k) the samples were air dried for 10 min; (l) the samples were amplified by universal primer of Potyvirus using the PCR protocol.

e) Gel cutting and purification

1. After the gel electrophoresis, the DNA bands were viewed under UV transilluminator.
2. Empty 1.5 mL micro centrifuge tube was weigh.
3. The DNA band was sliced with scalpel and transferred into the 1.5 mL micro centrifuge tube.
4. The gel slice was weigh and 3 volumes of QG buffer was added to 1 weigh go gel.

Calculation of QG buffer.

i) weigh of tube = 0.91 g

ii) weigh of tube + gel = 1.08 g

iii) weigh of gel = (ii) - (i) = 0.17 g

Therefore, the volume of QG buffer:

$$\frac{10\ uL}{0.01\ g} = \frac{x\ uL\ solution}{weigh\ of\ gel}$$

$$= 170\ uL$$

f) Gel extraction (DNA purification by centrifugation)

I. Dissolving the gel

1. 10 uL of membrane binding solution was added per mg of gel slice.
2. The sample was vortex and incubated at 50 - 65°C for 10 - 15 min or until the gel slice is completely dissolved.

II. Binding DNA

1. SV Minicolumn was inserted into collection tube.
2. Dissolved gel mixture or prepared PCR product was transferred to the mini column assembly.
3. The mixture was incubated at room temperature for 1 min and centrifuge at 16000 rpm for 1 min.
4. Flowthrough was discarded and reinsert mini column into collection tube.

III. Washing

1. 700 uL membrane wash solution (ethanol added) was added.
2. The tube was centrifuge at 16000 rpm for 1 min.
3. Flowthrough was discarded and reinsert the mini column into collection tube.

4. Step 1 was repeated with 500 uL membrane wash solution.
5. The tube was centrifuge at 16000 rpm for 5 min.
6. The collection tube was empty and recentrifuge the column assay for 1 min with micro centrifuge lid open to allow evaporation of any residual ethanol.

IV. Elution

1. Minicolumn was carefully transferred to clean 1.5 micro centrifuge tube.
2. 30 uL nuclease free water was added to the mini column.
3. The tube was incubated at room temperature for 1 min.
4. The tube was centrifuge at 16000 for 1 min.
5. Minicolumn was discarded and DNA stored at 4°C or 20°C.

g) Ligation (to prepare the recombinant plasmid)

1. All reagent and PCR product was mixed with micropipette and incubated overnight at 4°C. Longer incubation time will increase the number of transforming. Generally, incubation overnight at 4°C will produce the maximum number of transformants.
2. 2X Rapid ligation buffer should be vortex prior to use. The 2X Rapid ligation buffer contains ATP which degrades during temperature fluctuations. Multiple freeze-thaw cycles should be avoided and exposed to frequent temperature changes by taking single-use aliquots of the buffer.

h) Transformation

1. Competent *E. coli* (105 uL/tube) was leave in ice bath for 1 - 1.5 hours. Gently mixed it since competent *E. coli* is extremely fragile.
2. 50 uL of competent *E. coli* was added to ligated DNA (recombinant plasmid) and incubated in ice bath for 30 min.
3. The sample was placed in heat block at 42°C for 45 seconds and immediately transfer to ice bath for 2 min.
4. Aseptic technique was done under the laminar flow, 1000 uL S.O.C. medium was added into competent *E. coli* and the tube was wrapped with parafilm.
5. The tube was shaken (horizontally) at 150 rpm for 45 min at 37°C.
6. After shaken, the tube was centrifuge for 2 min to get pellet of *E. coli*.
7. The S.O.C. medium was poured off leaving about 200 uL of the medium in the tube.
8. The tube was vortex for about 15 sec.

9. In the laminar flow, the mixture was dropped onto LB medium and plate was spreader with sterile triangle rod until completely spread and dried. The plate was wrapped with parafilm.
10. The plate was incubated at 37°C overnight and transferred to 4°C for 1 - 2 days. The plate was leave upside down.

i) Culturing *E. coli* in TB medium

1. Five white colonies of *E. coli* were selected from the culture.
2. Using the sterile toothpick, a single colony was transferred into TB medium.
3. One blue colony was included as negative control.
4. The used toothpick was flamed afterwards.
5. The tube was wrapped with parafilm and incubated at 37°C and shaken overnight.

j) Mini Prep

Plasmid DNA was isolated and purified using LaboPass^{TM} Plasmid Mini Kit (*Hokkaido System Science, Co. Ltd. Japan and DNA Purification Kit, COSMO GENETECH*)

1. After shaking overnight, the tube was centrifuge at 3500 rpm (TOMY LC-100 Low speed centrifuge) for 7 min. The E. coli pellet was observed.
2. TB medium was poured off into separate flask and the pellet was kept (pellet size about 1 cm)
3. 250 uL buffer S1 was added (the tip should not touch the tube). In S1 buffer, RNAse must be added prior to use.
4. The pellet of E. coli was re-suspend and vortex for about 25 - 30 sec.
5. All suspension was transferred into 1.5 mL tube.
6. 250 uL buffer S2 was added (buffer S2 must be shaken before use) and then the tubes were inverted 3 - 4 times (do not vortex). Inverting the tubes will totally mix the cells with the buffer.
7. The tubes were incubated at room temperature for 1 - 5 min.
8. 350 uL buffer S3 (buffer S3 must be shaken before use) and 2 phases was appeared - clear and aqueous phase. It shows that the cells are already disrupted.
9. The tube were inverted gently 3 - 4 times.
10. The tubes were centrifuge for 10 min at 14000 rpm and E. coli was appeared at one side of the tubes.

11. Th DNA plasmid (clear solution) into the spin column. The plasmid has been trapped in the filter.
12. The tubes were centrifuge for 1 min at 14000 rpm.
13. The liquid poured off, 750 uL of buffer PW was added and centrifuge for 1 min at 14000 rpm. (PW buffer will further wash the filter to purify the plasmid).
14. The liquid was poured off, centrifuged for 1 min at 14000 rpm to completely dry the filter.
15. The spin column was removed and transferred it into 1.5 mL tube.
16. 50 uL of buffer EB was added into the centre of the filter and left at room temperature for 1 min. (The buffer EB will dissolved the plasmid from the filter).
17. The tube was centrifuge for 1 min and kept the DNA plasmid. (Plasmid is in the 1.5 mL tube and then the spin column was discarded).

k) Insert Check

1. 1 - 1.5% of agarose gel prepared.
2. 2 uL of loading buffer was mixed (6X load dye) and 2 uL negative control. The negative control is the blue colony.
3. 2 uL loading buffer and 2 uL sample was mixed separately.
4. Each sample was loaded onto the gel.
5. The gel was ran for 30 min.
6. The gel then was stained in EtBr for 3 min and rinsed with distilled water.
7. The bands were viewed under UV transluminator.

l) Cycle Sequence

Materials	For sequencing (uL)
q.s. (double distilled water)	2.0
Sequence buffer (Big dye Terminator 5X sequencing buffer	1.0
Primer (sp6/T7)	0.5
Premix	2.0
DNA (PCR product)	4.5
Total (uL)	10.0

Subject to Thermal Cycle: Sequence Program

1 - 24 cycle	step 1	96°C	10 sec
	step 2	50°C	5 sec
	step 3	60°C	4 min
25 cycle		4°C	forever

(about 3 hours)

m) Precipitation

1. After thermal cycle, 1.5 mL tube was prepared.
2. The sample (cycle sequencing product) used = 10 uL
   3M acetic acid = 1 uL
   99.5 - 100% EtOH = 30 uL

Optional step: The sample can be kept at -30°C for a long time or proceed to the next sequencing procedure.

3. The tube was mixed and centrifuge briefly.
4. The sample was put in ice block for 10 min.
5. The sample was centrifuge at 20°C about 20 min at 14000 rpm.
6. The supernatant was discarded and the pellet was kept.
7. 100 uL of 99.5% EtOH (not cold) was added and centrifuge for 5 min at 14000 rpm.
8. Step 6 was repeated.
9. 100 uL of 99.5% EtOH was added and rolled horizontally about 20 sec.
10. The tube was centrifuge for 5 min at 14000 rpm.

11. The supernatant was discarded and pellet was dried for 2 min at room temperature (open lid).
12. The tube was put in heat block at 95°C for 2 min (open lid).
13. Then, the tube was put in ice block for 5 min (close lid).
14. The pellet was kept at 4°C.

n) DNA analysis using Automate Sequencer (*Applied Biosystem 3130xl Genetic Analyser, HITACHI*)

1. The pellet was dissolved with 20 uL Hi-Di Formamide.
2. The sample was put into the plate carefully (should have bubbles).
3. The plate was put into the machine.

o) Sequencer machine

a. The computer and machine was turned on.
b. Program 3130xl data collection v30 was opened.
c. Program check (red to yellow to green light).
d. 1X Buffer EDTA was changed and used about 16 mL.
e. Polymer 3130 POP 7 was changed.
f. 1X buffer in the bottle was changed.
g. Bubble was discarded.
h. The program was ran and waited until the temperature goes to 60°C.

Note: a map must prepared based on the sample arrangement in the plate. 1X buffer EDTA was prepared by adding 3600 uL 10X EDTA buffer to 36 mL distilled water. All buffers were changed after using it for 1 week and washed and wiped with water. 3 containers are water and 1 with 1X EDTA buffer.

Results and Discussion

No. of well	Samples
M	100 bp ladder
1	Negative - Patrick
2	Passion fruit leaf - Patrick
3	Positive Poty - Patrick
4	Negative - Yatie
5	Passion fruit leaf - Yatie
6	Positive Poty - Yatie

Based on Figure 2, only one RNA band has been observed on the gel electrophoresis. The band was obtained from positive infection leaf sample of passion fruit. The results showed that the inoculated passion fruit sample was not infected with Potyviruses. The size of the RNA product is approximately 1700 bp. This size indicated that the RNA virus extracted from the passion fruit was belongs to the Potyviruses. The unsuccessful results showed by other sample are due to the failed during the sap inoculation (for inoculated sample), problem occurs during homogenisation of the sample (homogenized too hard or the sample was heated excessively for too long). Several troubleshooting options can be done to overcome the problem:

a. Focus on homogenisation step. Make sure all genomic DNA was sheared and release all the RNA from cell. Homogenate all the plant debris to reduce the lost of total RNA.
b. Reduce the degradation of RNA. The sample must be homogenised in burst of 30 - 45 sec with 30 sec of rest. Make sure all the sample was directly into contact with the liquid nitrogen and mortar and pestle used should be cold to inactivate the degradation enzyme.

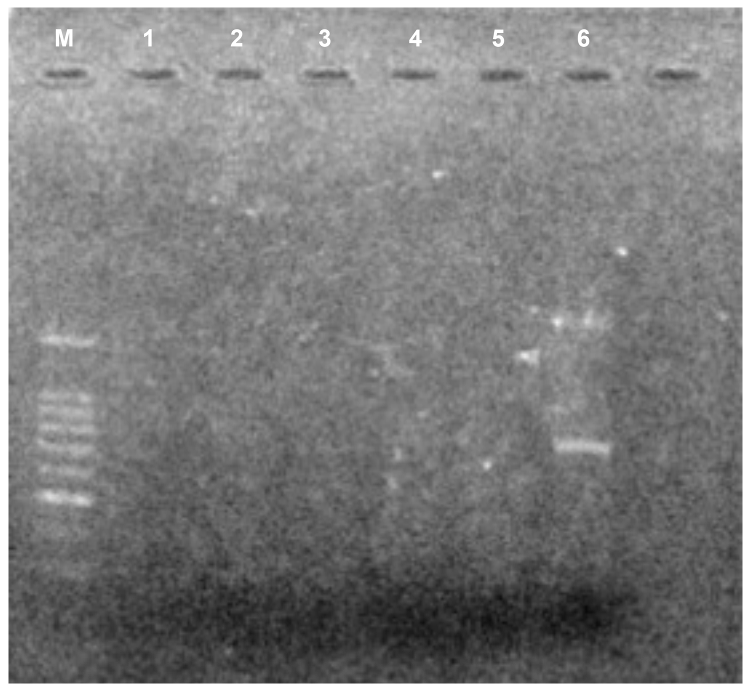


Figure 2: Electrophoresis of RNA from passion fruit infected plant (Potyviruses) on 1.5% agarose gel.

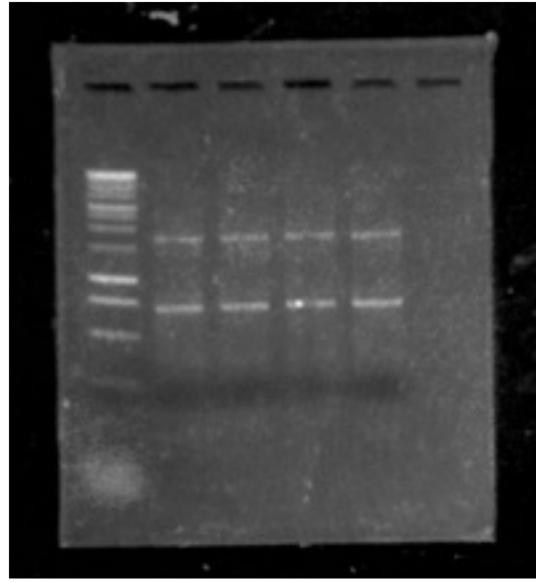


Figure 3: Gel electrophoresis of DNA from passion fruit infected

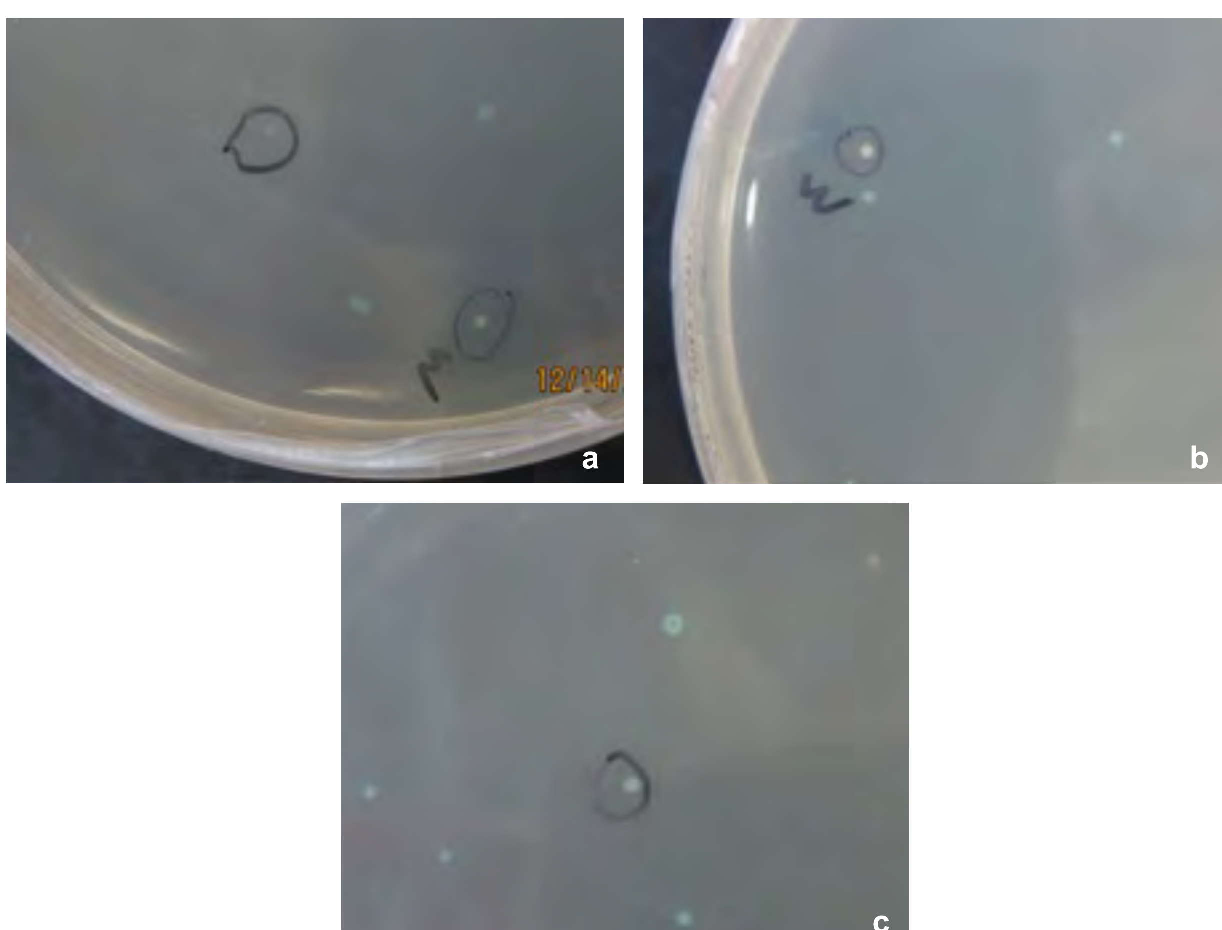


Figure 4: Colony of *E. coli* growth on TB media and was chosen for insert check procedure. (a) and (b) White colony labelled with circular mark are white colony which indicate to have virus insert; (c) Blue colony (not labelled) have no virus insert.

E. coli colony was grew on the TB media for more than 24 hours. The plate was maintained in the $4^{o}C$ refrigerator for 2 days to prevent overgrowth of the bacterial cell. Before insert check procedure was done, the colony should be observed carefully to make sure only white colony should be chosen. Plasmid DNA of E. coli was purified first before undergo insert check procedure.

Appendix 13

Detection of Protein using SDS-PAGE and Western Blot

Introduction

The principle of SDS-PAGE is based on the migration of charged molecules in the gel matrix in response to an electrical field. This method facilitates the separation and resolution of a mixture of protein according to the molecular weight. SDS-PAGE can carried out two ways; under reducing and non-reducing conditions.

Under reducing conditions, SDS-PAGE involves the linearisation of proteins by the dissociation of inter and intra chain disulphide bond and this can be achieved by heating the protein. Proteins are coated with negative charge in the presence of the anionic detergent in SDS detergent. The proteins were separated and resolved as discrete bands as they migrate in electric field. In order to separate the proteins, 2 types of polyacrylamide gel are used; stacking gel and separating gel. Proteins firstly will be 'stack' into the concentrated layer before entering the separating gel. This is due to the low percentage of gel (usually 3% w/v) of acrylamide are used through which proteins of all sizes readily migrate in electric field. However, different percentage of acrylamide is used in separating gel. The usual percentage of gel used is 5 - 7% w/v, appropriate concentration to resolve different molecular weight of proteins. In addition, the gel have larger pore size for the proteins to pass through and will optimally resolve large molecular weight proteins. Besides that, if the gel have high concentration of acrylamide (12.5 - 15% w/v), it have smaller pores which optimally resolve proteins in the lower molecular weight range.

Western Blot is also called immunoblotting. This technique is used for analysis of individual proteins in a protein mixture. Firstly, the protein mixtures will be applied to a gel electrophoresis (SDS-PAGE) to sort the proteins. The separated bands are then transferred to a carrier membrane (eg: nitrocellulose) which called blotting. The proteins in this immunoblot are accessible for antibody binding for detection. The function of antibodies are to detect target proteins on the Western Blot (immunoblot). The antibodies are conjugated with fluorescent/radioactive/enzymes that give a subsequent reaction with an applied reagents, leading to a colouring/emission of light, enabling detection.

Materials and Methods

(A) Preparation of gel

1. Equipments (glass plate, spacer and comb) needed for gel preparation were wiped with 70% ethanol.
2. The spacer was placed along the edge of the square glass.
3. Another square glass was placed on the glass assembled with spacer.
4. The glasses were held together by clips.
5. Two separate gels were made (8% separating gel and stacking gel) followed the recipe in Table 1 and Table 2, respectively.
6. The prepared separating gel was poured into the glasses, 2/3 from total amount.
7. 70% ethanol was poured onto the separating gel to prevent the gel from dried out and eliminate the bubbles.
8. The remaining separating gel was kept and left inside the beaker to be used as indicator of solidification of gel.
9. After the separating was solidified, the ethanol was removed and the surface of gel washed by distilled water and dried it with filter paper. Be careful, do not scratch gel surface.
10. After stacking gel was prepared, the gel was poured onto the separating gel and eliminate bubbles.
11. Then, the comb was inserted into space of glasses.

Table 1: Ingredients for 8% separating gel preparation

Ingredients	Amount (mL)
30% Acrylamide gel	5.3
1 M Tris-HCl buffer (pH 8.8)	5.0
10% SDS (w/v)	0.2
Demineralised water	9.3
Tetramethylethylenediamine (TEMED)	10 uL
10% Ammonium peroxosulphate (AP) (w/v)	0.2

*TEMED and AP was added final before gel pouring to prevent immediate solidification of gel.

*Percentage of separating gel is depends on the size of protein.

Table 2: Ingredients for stacking gel preparation

Ingredients	Amount (mL)
30% Acrylamide gel	1.5
0.5 M Tris-HCl buffer (pH 8.8)	2.5
10% SDS (w/v)	0.1
Demineralised water	0.1
Tetramethylethylenediamine (TEMED)	10 uL
10% Ammonium peroxosulphate (AP) (w/v)	5.8 uL

*TEMED and AP was added final before gel pouring to prevent immediate solidification of gel.

Samples were used from bovine protein (1 mg+900 mL distilled water), 10 dilution factors were done. Only dilution 1, 5 and 10 used for protein detection.

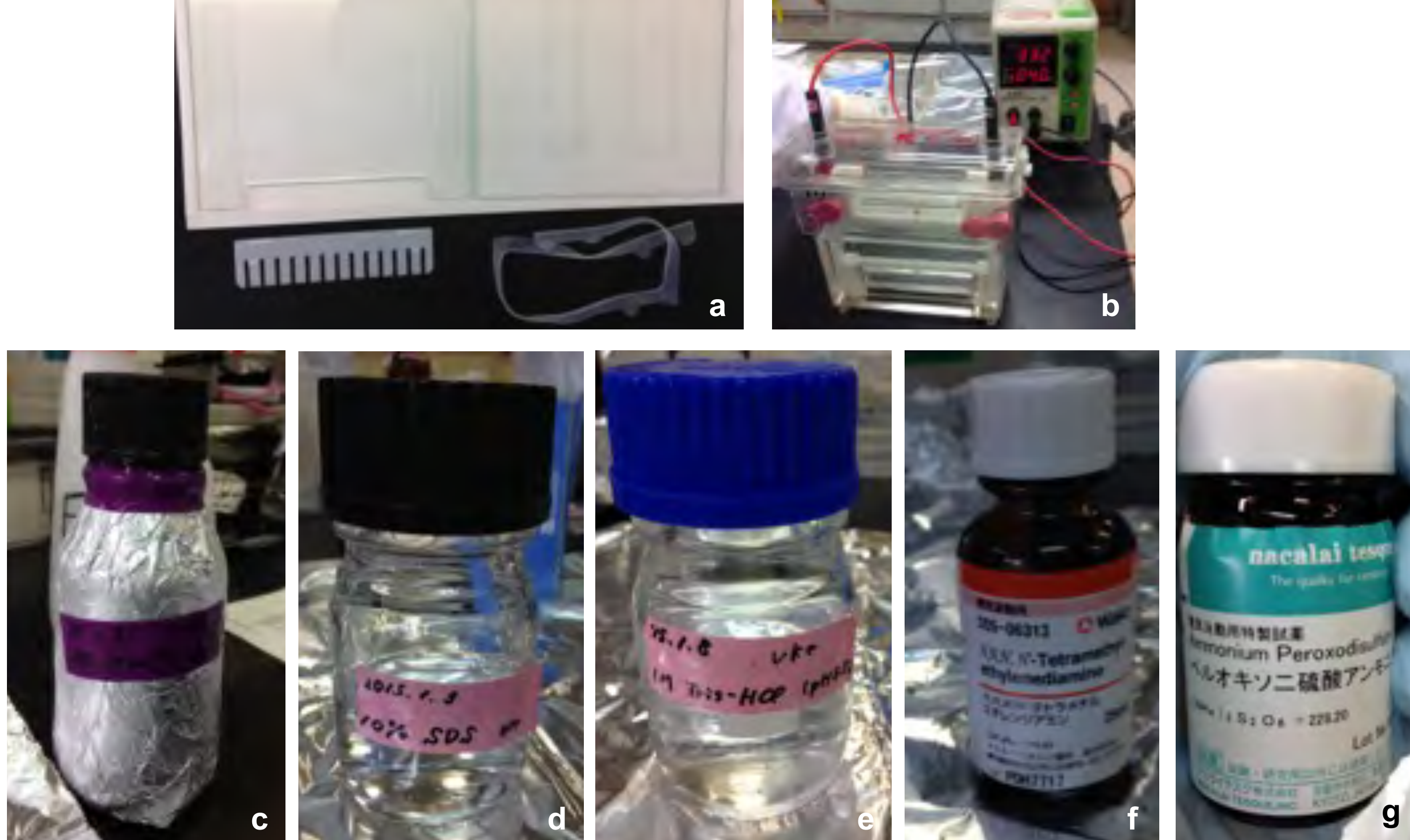


Figure 1: Equipments and reagents used for gel preparation. (a) Equipments to make gel (square glasses, comb and spacer); (b) gel electrophoresis & electric supply; (c) 30% acrylamide solution; (d) 10% SDS; (e) 1 M Tris-HCl (pH 8.8); (f) TEMED; (g) Ammonium peroxodisulfate.

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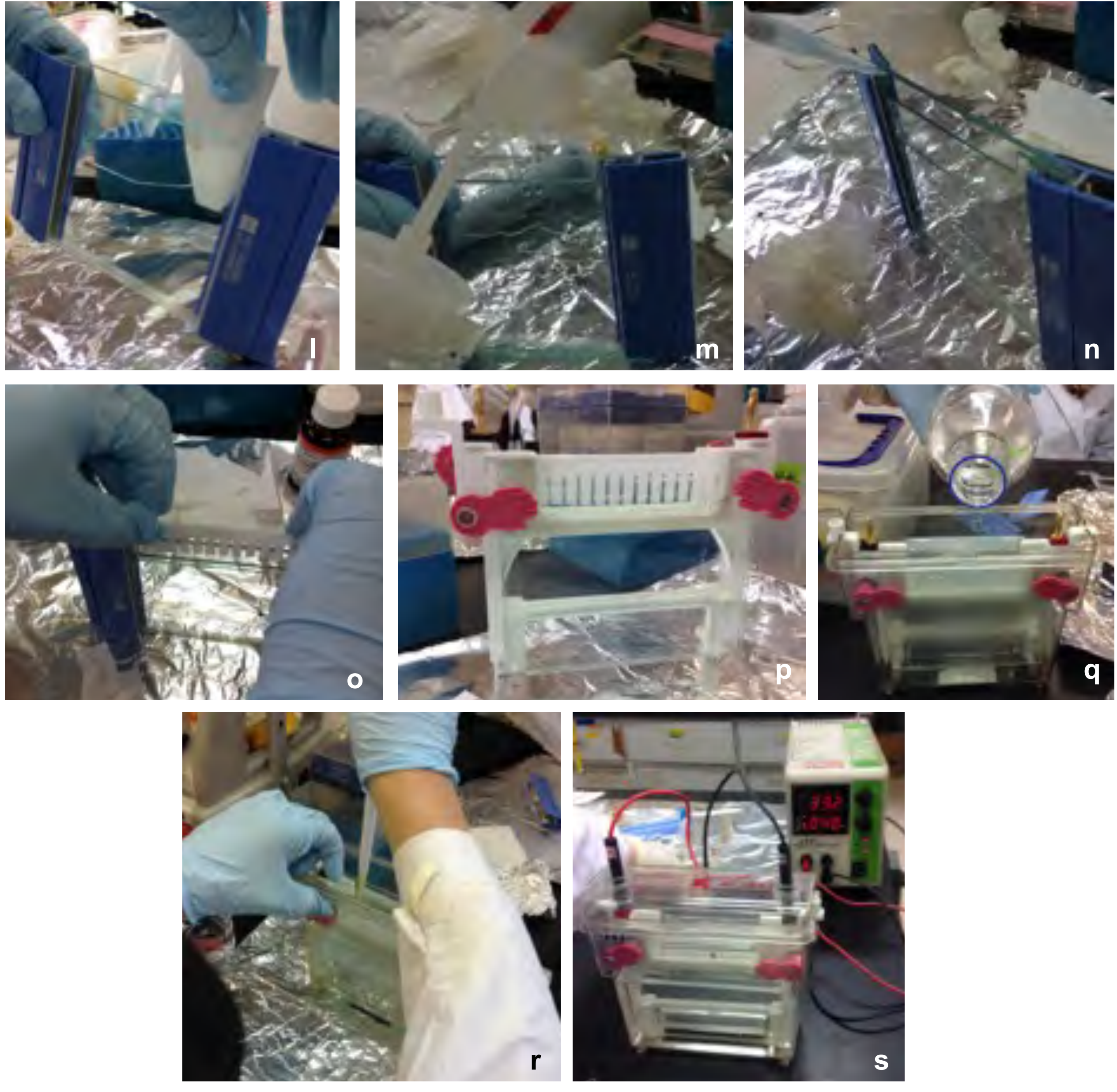


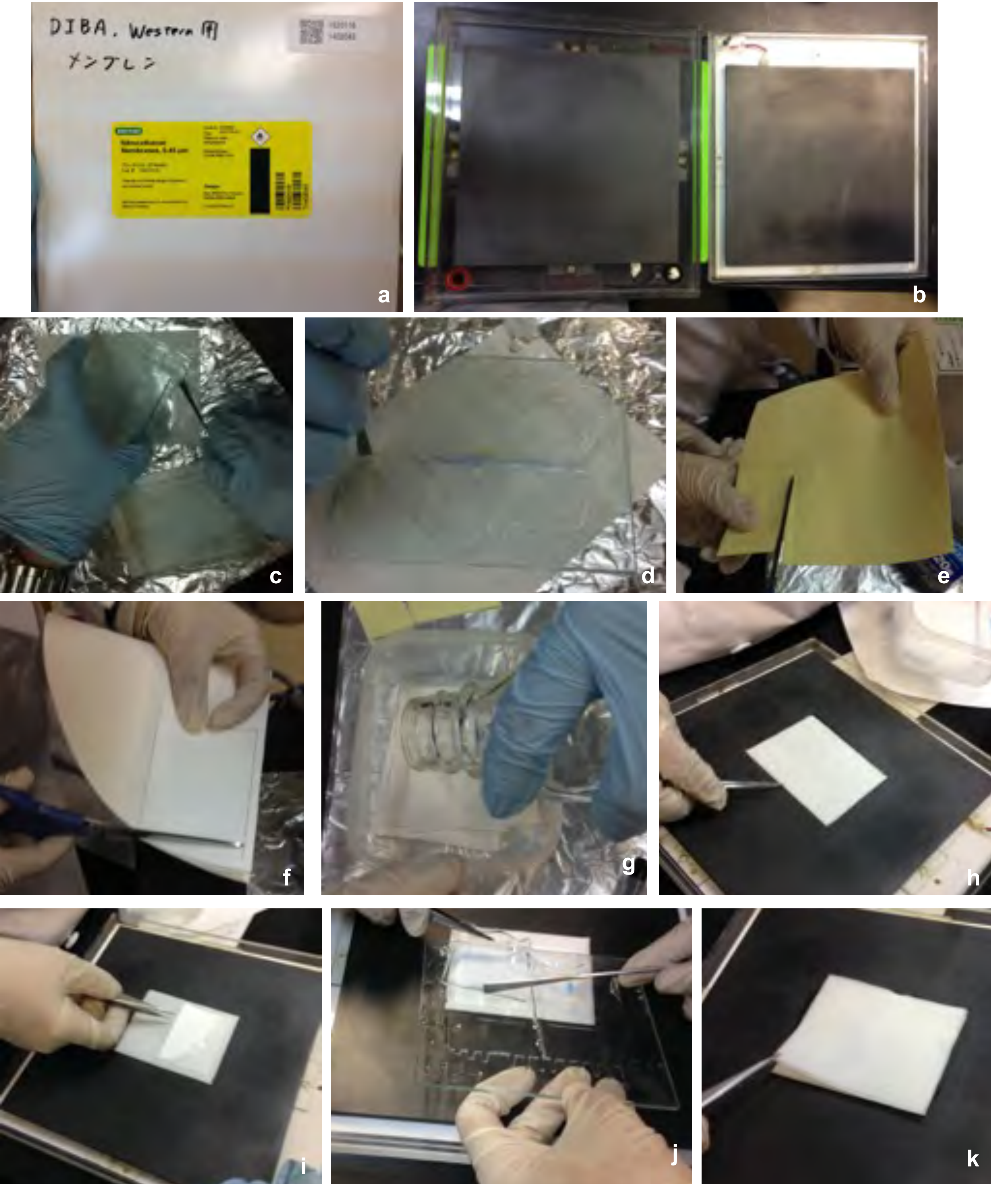
Figure 2: Procedures to make the gel. (a) Disinfected with 70% alcohol; (b) and (c) assembled all the equipments; (d) 30% acrylamide gel added into the beaker; (e) additional of 10% SDS; (f) The reagents were mixed with distilled water; (g) The solution was stirred; (h) Additional of 10% AP; (i) Additional of TEMED; (j) The gel was poured into the gel case; (k) Ethanol was added into the gel; (l) ethanol was removed using filter paper; (m) separating gel was rinsed with distilled water; (n) stacking gel was poured onto the separating gel; (o) The comb was placed into the gel; (p) The solidified gel was assembled with electrophoresis apparatus; (q) Electrophoresis buffer was poured into electrophoresis tank; (r) sample was loaded into the well; (s) Electrophoresis was started.

(B) Electrophoresis

1. The gel was set into the electrophoresis tank.
2. Electrophoresis buffer was added into the tank.
3. All bubbles under the gel must be removed.
4. The comb was removed carefully.
5. The shape of the well was adjusted with the syringe.
6. Sample and loading dye was mixed well (10 uL loading dye+10 uL sample).
7. The sample was heated in 100°C for 5 min.
8. The sample and marker was loaded into the well.
9. The electrophoresis was ran firstly using 20 mA for 10 min. Then, the voltage was increased to 40 mA for 30 min after the top of the electrophoresis was exceeded stacking gel.

(C) Western Blot Protocol

1. The western blot membrane was cut same size as the gel.
2. The filter paper was cut 1 cm more from the size of the gel (6 pieces of filter paper required for each gel).
3. The filter paper was soaked in transfer buffer.
4. The transfer device was put according to this order: filter papers (3 pieces), gel, membrane and filter papers (3 pieces).
5. All the air between paper and membrane was pushed out using small roller.
6. The voltage of machine was set up based on the surface area of the gel (eg: surface area of the gel = 12 cm^2, therefore the voltage should be used = 24 mA). The procedure was ran for 1 hour.
7. After 1 hour, the membrane was soaked in blocking solution and shake for another 1 hour.
8. The membrane was rinsed with distilled water and dried out. The protein detected on the membrane was observed.
9. The procedure was stopped up to this stage because the unavailability of antibody.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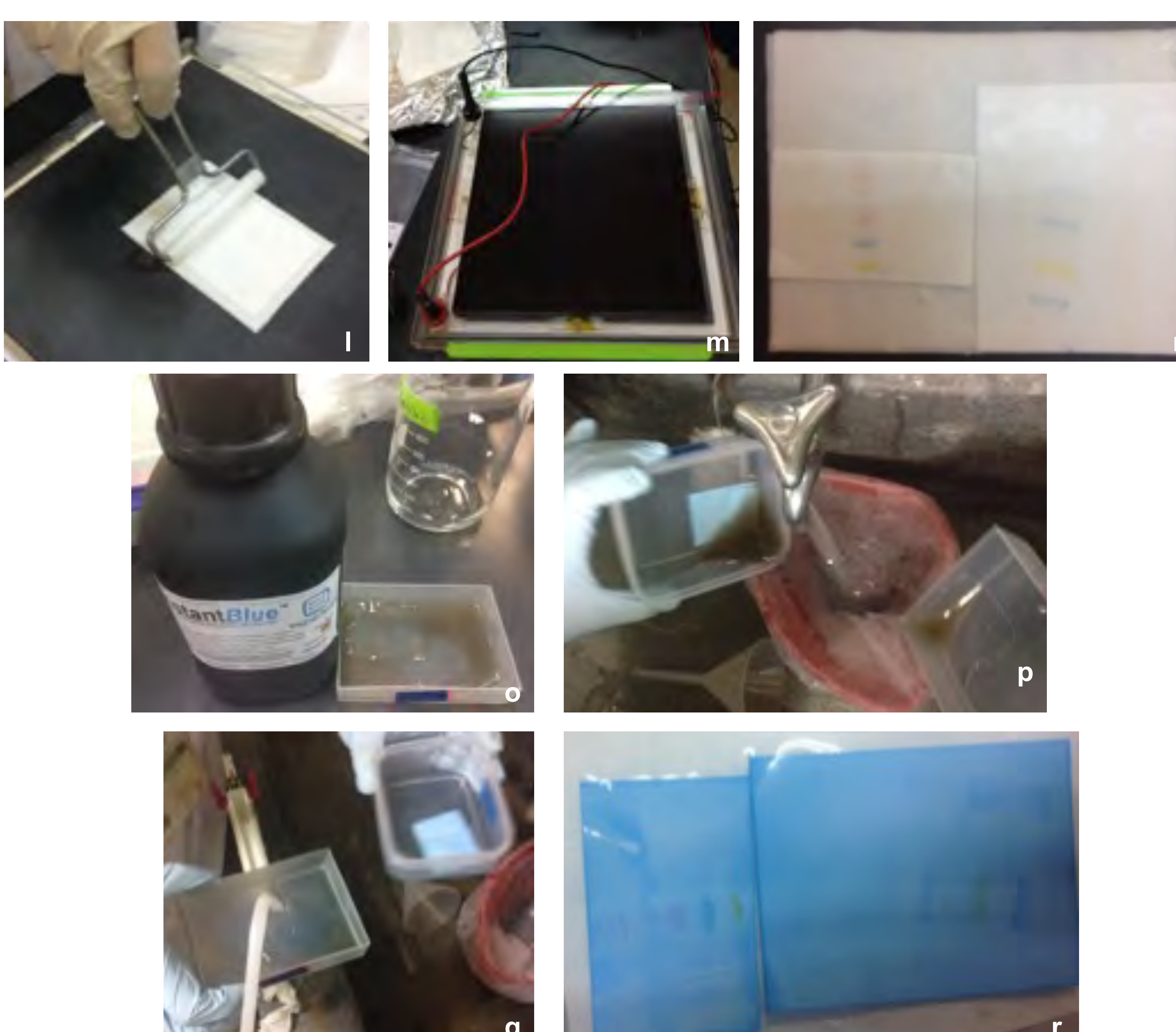

Figure 3: Western Blot procedure. (a) Membrane for Western Blot test; (b) Western Blot divide; (c) Gel was taken out from squared glasses; (d) Gel was cut to separate the protein which only content protein bands; (e) Membrane was cut according to gel size; (f) Filter paper; (g) Filter papers were soaked with transfer buffer; (h) 3-pieces of filter papers were first arranged on Western Blot device; (i) The membrane was placed on the filter paper; (j) The gel was transferred onto the membrane; (k) Final layer, the filter papers were covered the gel; (l) Roller was used to eliminate bubbles between membrane and get layer; (m) The gel was ran for 1 hour; (n) After 1 hour, the gel was removed from the membrane; (o) Gel and membrane was stained using Instant Blue Staining; (p) The stains was poured off; (q) Gel and membrane was rinsed using distilled water; (r) Dried the membrane and result was observed.

Results and Discussion

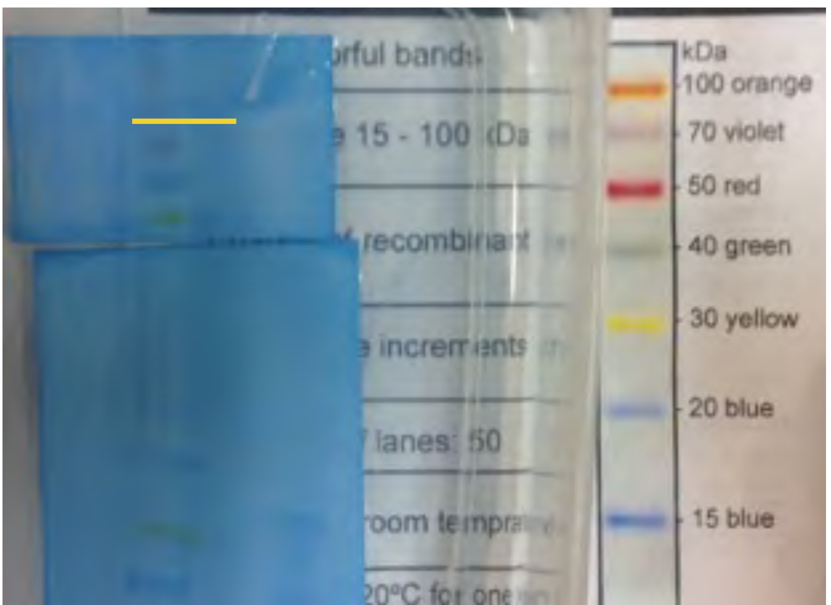

Figure 4: The band was appeared on the membrane after Western Blotting procedure.

Based on the result from Figure 4, the band is not obviously embedded on the membrane after Western blotting procedures. The band of the protein was detected from 1st dilution of bovine sample. The protein obtained have approximately 50 Dalton in length. The proteins are not stacked and separated properly during the gel electrophoresis. Other samples was failed to detect on the membrane due to suboptimal protein problem. This is the common condition happen with Western blotting and can due to many different problem:

a) Insufficient protein. Increase the amount of total protein loaded in the gel.
b) SDS-PAGE gel incorrectly made or run.
c) Improper sample preparation for gel loading. Proper migration depends on protein samples containing SDS/dithiothreitol (DTT)/2-mercaptoethanol must be heated prior to loading. Use pertained protein to monitor transfer.
d) Insufficient antibody. Increase antibody concentration.

Conclusions

SDS-PAGE is a useful method to detect the large molecule proteins especially virus proteins together with western blotting. The proper preparation of gel, sampling preparation and loading, type of membrane used, voltage of gel electrophoresis and the correct concentration of antibodies should be monitored to get good quality results.

Reference

Gomes, A. V. (2009). Western blotting tips and troubleshooting guide. NPB Department, University of California Davis. (Retrieved from: http://www.medschool.vanderbilt.edu)

Appendix 14

Extraction of dsRNA from Cucumber mosaic virus (CMV) Infected Plants

Introduction

Cucumber mosaic virus (CMV) can infects more than 1200 species over 100 plant families and cause significant losses in vegetables and horticultural crops. CMV causes systemic infection in most plants and remain symptomless. The symptoms may varies depends on the crop infected and the age of plant infection occurs. Almost all species of Cucurbits can be infected by CMV. However, the severity of infection may vary. Plants which infected at early season show malformed leaved, severely stunted, fruit unmarketable due to the pronounced rugosity on the fruit surface. For example, CMV infected watermelon will cause severely stunted growing tips. For squash, the fruit shows colour breaking (green blotching patterns). Common symptom of CMV infection is mosaic pattern on fruits and foliar.

CMV is the type species of the genus Cucumovirus in the Family Boromviridae. CMV are consist of 3 spherical particles. The CMV genome consists of 3 single stranded and messenger-sense RNA molecules. This messenger-sense RNA molecules have RNA-1 (~3350 nucleotides), RNA-2 (~3050 nucleotides) and RNA-3 (~2200 nucleotides). Each molecule is enclosed within a protective protein coat with each being a distinct single spherical-shaped particle. RNA-3 particle may contain forth RNA strand encodes the coat protein gene from which the CMV coat protein is produced.

The isolation and properties of viral specific double-stranded RNA (dsRNA) from tissue infected with RNA viruses was well documented. The quality of dsRNA is different for some viruses. The demand for better recovery and quality of dsRNA was resulted in the development of simple and rapid column/batch procedure for isolation of dsRNA. This method was based on affinity of cellulose powder for nucleic acid which specifically the adsorption of dsRNA at 15% of ethanol concentration. The product will analysed by gel electrophoresis and dsRNA identified by ribonuclease treatment. This technique is a useful tools to study the RNA virus replication and for detection and diagnosis of virus directly from infectious tissue.

Materials and Method

1. 5 g of frozen leaf sample (Cucurbit: Karasu uri) was weigh.
2. The sample was grounded with cold mortar and pestle to a fine powder and was homogenised with 10 mL of 2X STE buffer.
3. The sample mix was transferred into homogeniser tube and 50 uL of 2-mercaptoethanol was added.
4. Following reagent mixtures were prepared [2.5 mL phenol, 2.5 mL chloroform, 0.1 mL isoamyl alcohol (3-methyl 1-butanol/ pentanol)] in the pestle with remaining grounded sample. The mixtures were transferred into homogeniser tube containing sample.
5. The sample was homogenised for 5 min at 5000 rpm.
6. The mixture was then centrifuged at 10000 rpm for 10 min.
7. After the mixture was centrifuged, the supernatant was transferred into measuring cylinder and the volume of supernatant was recorded to determine the amount of ethanol to be added later. The maximum volume of ethanol can be added was 15%. Amount of ethanol was calculated using following formula: [vol. of supernatant/0.85] - vol. of supernatant.
8. The sample was incubated at 4°C for 60 min.
9. After incubation, the sample was centrifuged at 10000 rpm for 10 min and supernatant was transferred into glass tube.
10. After centrifugation, 1 g of CF-11 cellulose powder (Whatman) was added to the supernatant and the mixture was vortex for 15 min with 30 sec intervals.
11. The powder was placed in a glass column and washed with 100 mL of 15% ethanol / 85% STE buffer. The dsRNA was eluted with 20 mL of ethanol-free STE buffer.
12. The eluate was then mixed with 0.8 g of CF-11 cellulose powder and 3.6 mL of 99.5% ethanol and subjected to a second cycle of chromatography.
13. The second column was washed with 100 mL of 15% ethanol/85% STE.
14. Finally, the dsRNA was eluted with 16 mL of STE buffer (separated into 2 tube, 8 mL each tube), made to 30 mM $MgCl_2$. 2 uL DNase I was added into each 8 mL sample.
15. Both tubes were incubated in warm water (30°C) for 30 min to eliminate all DNA.
16. 16 mL of 99.5% ethanol was added into the sample.
17. Samples were stored at -20°C overnight or 80°C for 1 hour.
18. After storage overnight, the sample was centrifuged in cold condition (0°C) at 10000 rpm for 15 min. Thee supernatant was discarded and pellet was kept (clear pellet).
19. The sample was left to dry on filter paper to remove remaining ethanol. Then, the tube was put upside down inside the dryer for 5 min.

20. 100 uL of 10X loading dye was mixed with 500 uL RNase free water. The sample was mixed vigorously with this solution (50 uL+sample).

21. The tube was spin down for few second to collect the sample and transferred into 1 tube (PCR tube). Total sample collected was 100 mL.

Preparation of 2X STE Buffer

Total volume: 1 L

NaCl = 11.688 g

Tris-HCl = 12.114 g

EDTA-2Na = 0.7448 g

RNase free water = 800 mL

*Adjust pH = 7.0

Preparation of 1X STE Buffer

2X STE buffer + RNase free water

1 : 1

*mixed and shaking

Preparation of 1X STE buffer, 15% ethanol

2X STE buffer = 100 mL

RNase free water = 70 mL

*shake and mix, then add 100% ethanol = 30 mL

Preparation of Polyacrylamide Gel

1. All gel apparatus was assembled.
2. 7.4 mL double distilled water was added into the beaker.
3. Following reagents were added [1 mL 10X TBE buffer, 30% acrylamide: 1.7 mL, 10% APS: 100 uL, TEMED: 6 - 8 uL] and mixed vigorously. Then, the gel was poured into gel case.
4. The gel was left to solidified for 30 - 40 min at room temperature under light.
5. After the gel was solidified, 1X TBE buffer was added inside gel case. Middle of the case must be fully poured with the buffer.
6. The samples were loaded into each wells [1kbp marker=10 uL; sample= 6 uL).
7. The gel was ran for 50 - 60 min using 20 mA electricity.

8. Finally, the gel was stained with Ethidium bromide (30 - 60 sec), rinsed with distilled water and viewed under UV transluminator.

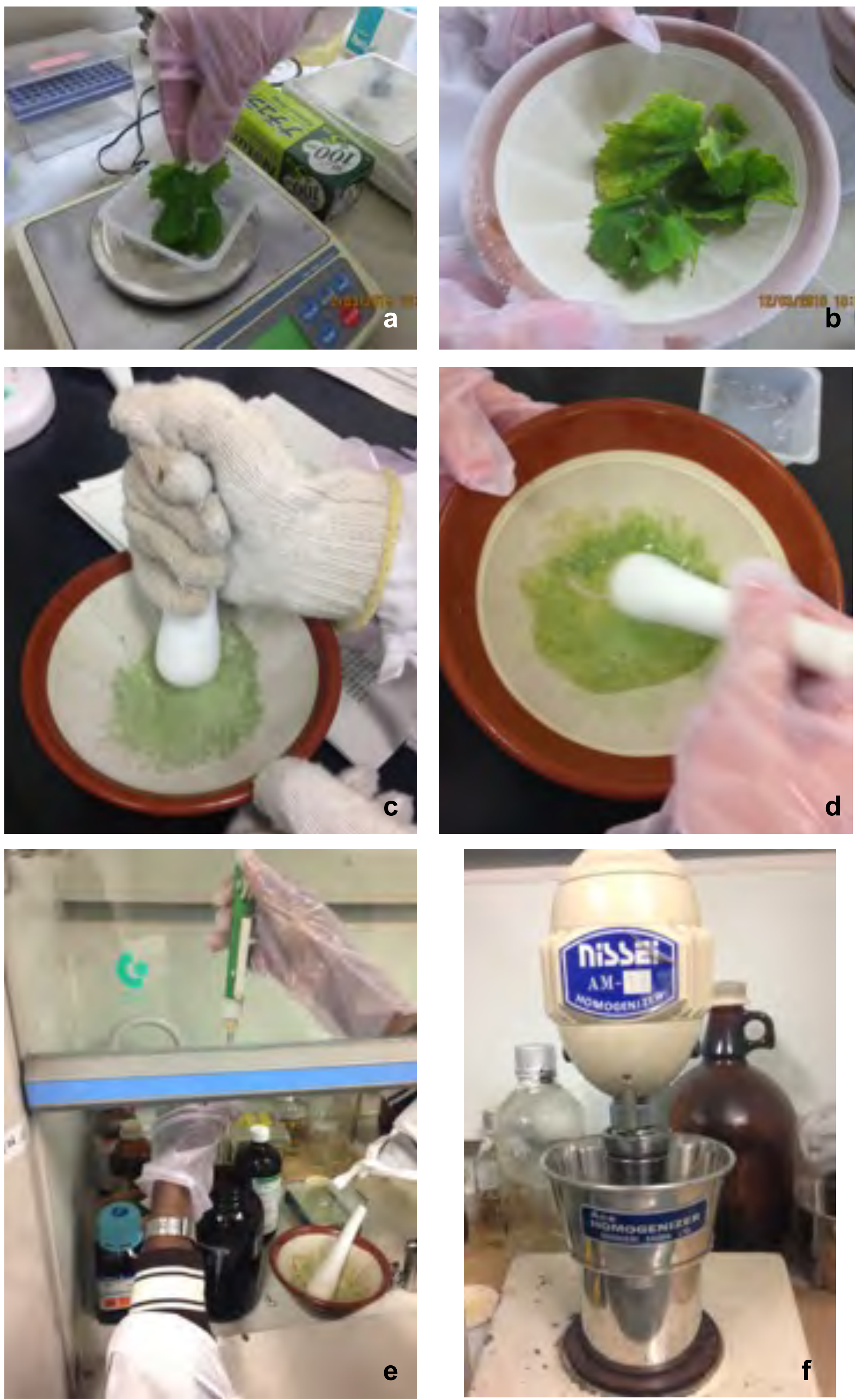

Figure 1: Procedure of dsRNA extraction. (a) weigh of infected leaf sample; (b) leaf sample was placed into cold pastel; (c) sample was grounded into powder; (d) sample was homogenised with STE buffer; (e) phenol, chloroform and pentanol was added into remaining sample; (f) mixture was homogenised using homogeniser.

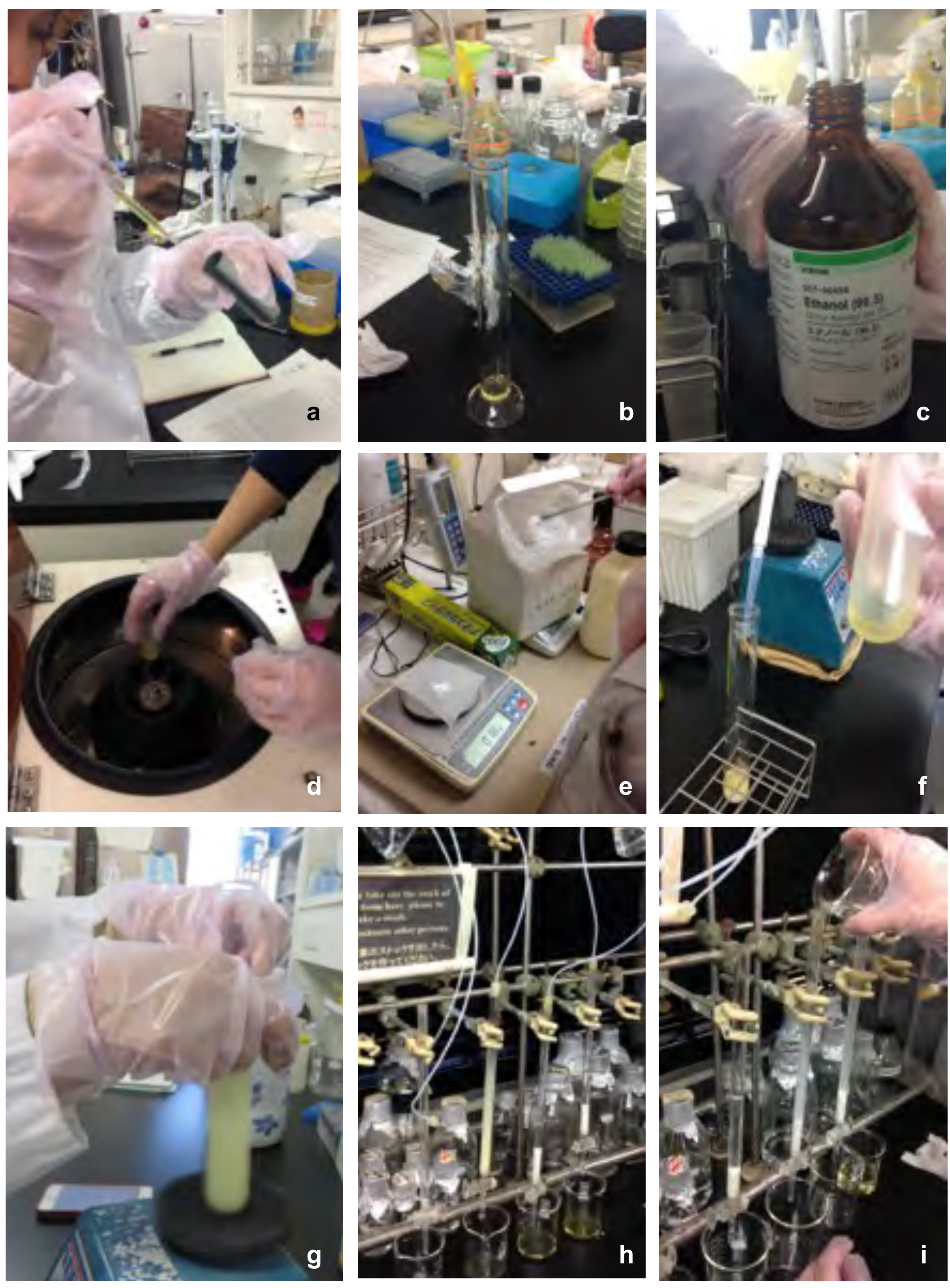

Figure 2: Procedure of dsRNA extraction. (a) supernatant was transferred; (b) volume supernatant was measured with measuring cylinder; (c) ethanol was added into the supernatant; (d) sample was mixed and centrifuged; (e) CF-11 cellulose powder was weigh; (f) supernatant was added into cellulose powder; (g) solution was vortex for 15 min with 30 sec intervals; (h) solution was washed with ethanol-STE buffer using chromatography tube; (i) additional of ethanol free-STE.

Figure 3: Procedure of dsRNA extraction. (a) additional of DNase into the sample; (b) additional of MgCl; (c) sample was incubated in the warm water; (d) additional of ethanol; (e) samples were dried on filter paper; (f) samples were added with dye; (g) sample was mixed vigorously; (h) samples were transferred into PCR tube for storage.

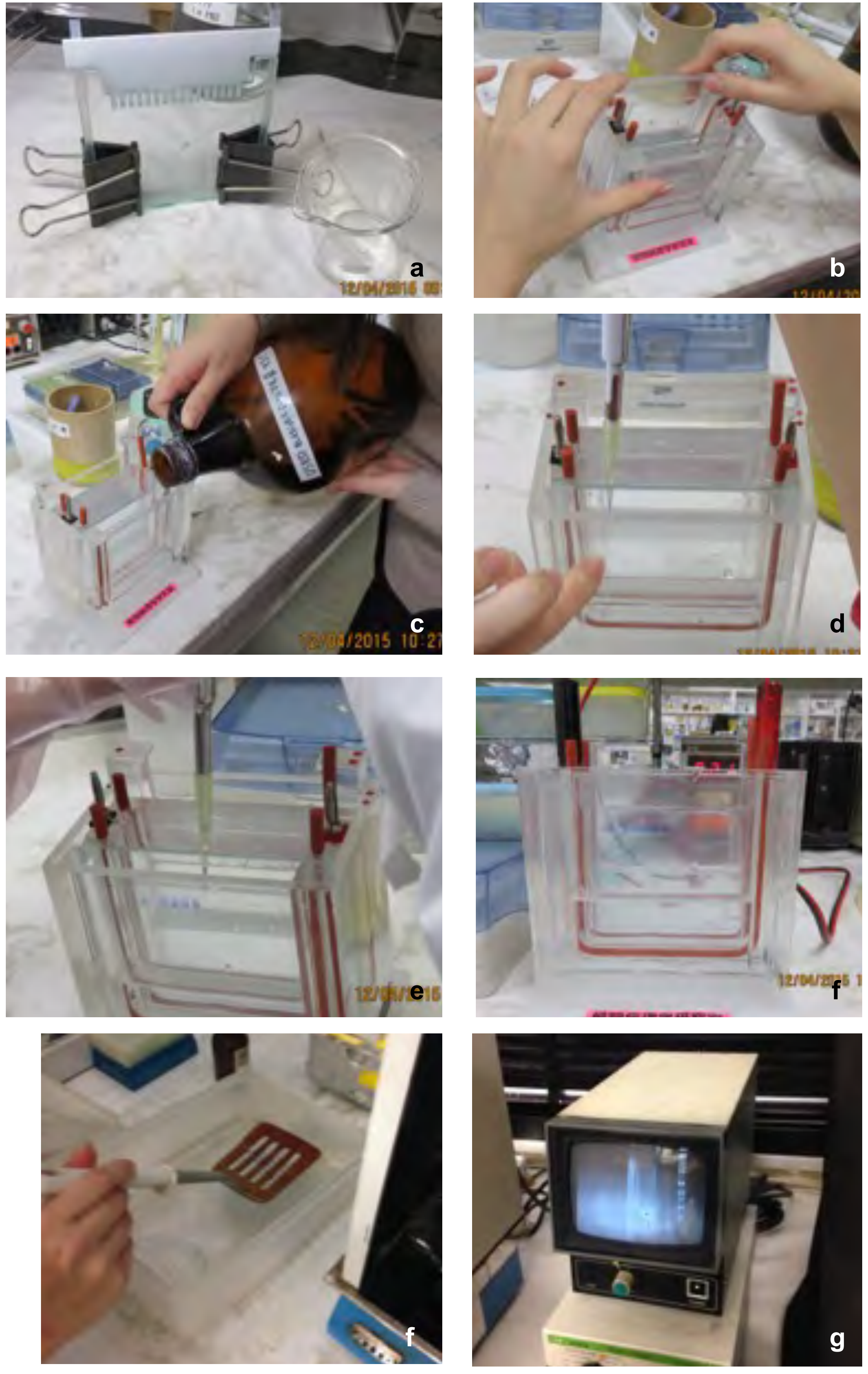

Figure 4: Procedure of dsRNA extraction. (a) polyacrylamide gel setting; (b) after gel was solidified, the gel was placed inside the main case; (c) 1X TBE buffer was added into the gel case; (d) 1 kbp marker was loaded into the first well; (e) all samples were loaded into each well; (e) gel was ran for 50-60 min; (f) gel was stained using EtBr and rinsed with distilled water; (g) gel was viewed in UV transluminator.

Results and Discussion

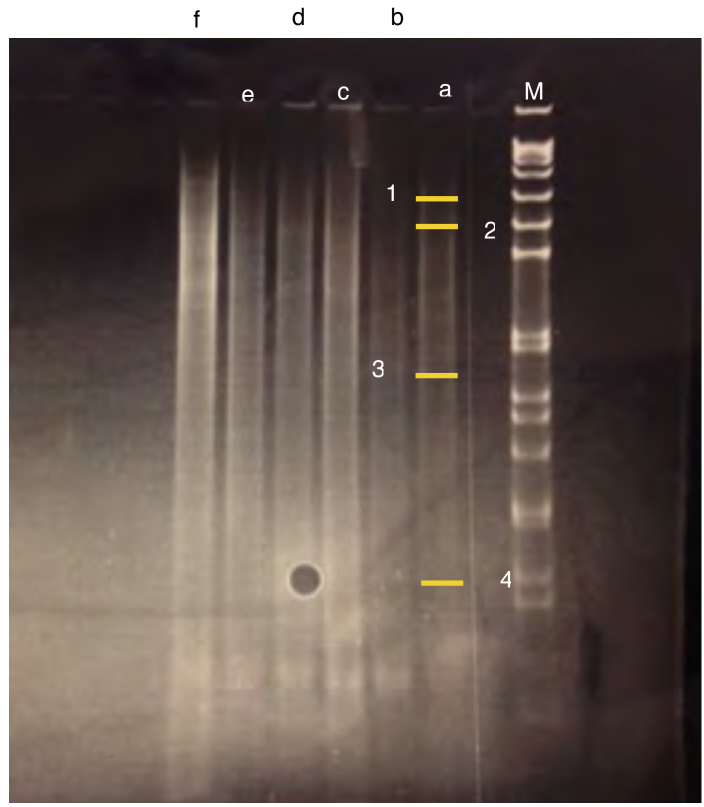

Figure 5: Electrophoresis of dsRNA from Cucurbit infected plant (Cucumber mosaic virus - CMV).

No. of well	Samples
M	1 kbp ladder
a	CMV - Yatie
b	CMV - Patrick
c	CMV - Ayumi
d	CMV - Chien
e	CMV - Fitri

Based on Figure 5, the result was showed that the cucurbit plant infected by Cucumber mosaic virus due to the detection of RNA 1~4 which is the unique characterisation of dsRNA of CMV. CMV is type member of the cucumovirus group, contains 4 positive sense single-stranded (ss) RNA species designated RNA 1~4. The bands obtained were faint due to the several reasons. There are:

a. The concentration of CMV is low in the infected plant. Highly infected plant leaf with visible/ severe symptom will accumulate high concentration of virus particles.

b. The proportion of sample and extraction buffer is not enough to get more dsRNA. Recommended proportion of sample to buffer is 1:1 or 1:2. For example, 1 g sample: 10 mL buffer or 1 g sample: 20 mL buffer.
c. The exact amount of supernatant should be measured accurately. Therefore, the correct amount of ethanol (15%) must be added into the supernatant to obtain more dsRNA. However, the amount of ethanol added should not exceed 15% from total supernatant measured.

Conclusion

Double stranded RNA (dsRNA) extraction protocol is one of the technique can be used to detect and identify specific viruses accurately. This technique is a powerful method for identification of Tobacco mosaic virus (TMV) and Cucumber mosaic virus (CMV). The absent of Poly A region is the unique characterisation of these viruses to differentiate them from other virus groups. For detection of these dsRNA bands, polyacrylamide gel was used instead of agarose gel because the gel able to separate the dsRNA molecule which have less than 500 bp in size.

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Appendix 15

Visit to Yokohama Plant Quarantine Office and Research Centre, Yokohama Plant Quarantine Section

Plant quarantine department plays an important role in order to protect country from introduction of foreign dangerous pathogens and diseases through international trade including import and exports of agricultural products from overseas. Therefore, the enforcement of plant quarantine procedures on each of products related to agriculture is vital especially to Japan which surrounded by seas and its highly potential to be invades by pests.

Yokohama Plant Protection Station is one of the plant quarantine office in Japan which have responsibility in implementations of quarantine procedures and protect Japan's agriculture industry from dangerous threats both from local and international trade. These quarantine procedures include import quarantine to prevent the introduction of overseas plant pests, export quarantine in response to requests from other countries and domestic quarantine to control pests in Japan. Major pests requiring precautions by the Plant Protection Station are Mediterranean fruit fly (Ceratitis capitate), fire blight (Erwinia amylovora), Codling moth (Cydia pomonella), Tobacco blue told (Peronospora tabacina).

Plant quarantine system in Japan can be divided into International Plant Quarantine and Domestic Plant Quarantine. Import quarantine include import plant inspection, post-entry quarantine and pre-shipment in country of origin. Meanwhile, export quarantine consist of export inspection and field inspection of export plants.

Implementations of plant quarantine procedures for imported products

There are 3 conditions of of items for importation:

a) Prohibited Items - The items are recognise could cause serious damage if they were introduce into Japan and detection of the the pests are difficult especially at entry points. Therefore, the importations of plants that may have such pests from the countries in which the pests are found is prohibited. Example of pests; i) Mediterranean fruit fly (mango, citrus fruit and kiwifruit), ii) fire blight (pear, loquat and Pyrachanta).
b) Items subject to quarantine - The items (plant and plant products that are not prohibited include seedlings, ornamental plants, cut flowers, bulbs, seeds, fruits, vegetables, grains, beans, woods, spice crops, ingredients for Chinese medicine etc. must be inspected when imported.

c) Un-restricted Items - The products have not undergo high degree of inspection and pose no threat of pest presence. Eg. wood products and processed tea.

Import quarantine also conducted prior to custom clearance at the place of entry including cargo inspection at seaports and airports, inspection of hand luggage and inspection of postal items.

For quarantine of seeds and seedlings, details inspection should be conducted because infected seeds cannot be detected during import inspections, Therefore, the imported seeds must be taken to the laboratory, where the are subjected to blotter tests and other thorough inspections. Cultivating of some seeds for certain period of time such as bulbs, fruit tree seedlings and scions and potato family for detection of viral diseases.

There are procedures for lifting import bans which applied to the prohibited items may allow for importations with certain conditions if the country of origin has technologies for disinfesting the items and framework for using these technologies properly and reliably. For example, the importation of mango from Malaysia is prohibited. However, the import bans are lifted after Malaysia was implements the infestation procedures for mango using Vapour Heat Treatment (VHT) under inspection of 2 quarantine officers from Japan.

Implementing quarantine in response to requests from Japan's trading partners:

1. Quarantine when exporting - services offered: a) collection and sharing of information on plant quarantine requirements of foreign countries, b) on-site export inspection at consolidation areas, c) technical training and lectures on pest control, fruits sorting etc for pests regulated by import countries.
2. Quarantine during cultivation - importing countries have requested Japan to certify that no pest infestation occurred during cultivation of seeds and seedlings.

Japan's Domestic Quarantine

Plant Quarantines stations implement various measures to prevent the spread of pests within Japan including inspections of seed potatoes and major fruit-free seedlings and regulating the movement of seedlings from regions with pest outbreaks to those without pests.

Inspection on supply of pest-free seeds and seedlings

Plant quarantine officers inspect national-designated seeds and seedlings for pests during their growth in order to secure the supply. Currently, they are inspected the potatoes seeds for viral diseases and ring rot.

Prevention from regional pest outbreaks

There are several pests found to be exists only in Nansei Islands (Okinawa Islands, Amami Islands and Tokara Islands) such as Giant African snail, Sweet potato weevil and Asian citrus psyllid. Plant Quarantine Station employs the quarantine procedures to eradicate and prevent the movement of the pests from other areas. If the new pests are introduced into an area, immediate detection and eradication steps must be apply to eradicate them at early stage. Therefore, they are closely cooperate with Japan Post Office that conduct customs clearance procedures and prefectures pest control stations to do continuous monitoring surveys to detect new pests and engage in emergency eradication.

Research on highly advanced plant quarantine

Plant Protection Station also conducting research on plant quarantine pests in order to improve the plant quarantine system, obtain new information of pests and diseases from around the world and develop new inspection techniques for unknown pests biologies and impacts on plants by bringing them from their origin countries.

Research on phytopathogens

Plant Protection Stations collect information from overseas on epidemiology, biology, prevention methods and others for plant diseases which have not been infested on plants in Japan. The researchers will take samples of pathogens into Japan and investigate their morphology, biochemical properties, serological properties and molecular biological properties in order to develop inspection procedures and identification methods on the pests.

Development of disinfestation technologies

Development of precise and safe disinfestation methods are the vital part of plant quarantine. Therefore, research has been done to develop treatment technologies including chemical and physical disinfestation on the pests.

Maintaining quarantine data

Plant Protection Station also develop the database system for inspected plants and their countries of origin or intercepted pests which is essential for plant quarantine officers and stations to conduct the on-site quarantine effectively. The system contains statistical data

on plant quarantine which includes in publications such annual reports. These database are released in the website and easily can be access by public.

Other than that, Research Centre also provide training in variety of specialised fields include botany, applied zoology, entomology, plant pathology, agricultural chemicals, sterilisation techniques, plant quarantine administration and trade practices. Cooperation with other countries for identification of alien species regulated as Invasive Alien Species Act and in genetic diagnosis. They also develop the system which simplify the procedures and improve conveniency of application certain application/declaration for export and import products by companies from local and overseas such as Phytosanitary certificates.

Yokohama Plant Protection Station

The station consist of several divisions situated in Yokohama city include:

1. General Affairs Division - General Affairs Section, Accounting Section
2. Research Division - Planning and Coordination Section
3. Plant Protection Operation Division - Import Quarantine Section (Ship Cargo), Import Quarantine Section (Sea-container Cargo), Import Quarantine Section (Seed and Seedling), Export Quarantine Section, Domestic Quarantine Section and Pest Identification Section, Post-entry Quarantine Section (in Tsukuba Centre).
4. Plant Quarantine Training Centre.

For Plant Quarantine Research Centre, they have 6 section which conduct researches on pests and diseases, pest risk analysis and plant quarantine treatment. The sections include:

1. Disinfestation Technology Section
2. Entomology and Nematology Section
3. Pest Risk Assessment and Pest Risk Management Section
4. Living Modified Organism (LMO) team
5. Pest Identification Section

Figure 1: Some of the plant quarantine pests for imported agricultural products from overseas prohibited by Japan’s Plant Protection Station

Figure 2: Plant Protection Research Centre. (a) Briefing about the research centre by quarantine officer; (b) Research laboratory for Oriental fruit fly; (c) Research laboratory for quarantine treatment; (d) Analytical equipments for detection of agriculture chemicals; (e) Living Modified Organism (LMO) DNA extraction laboratory; (f) Pest Risk Analysis workflow; (g) Briefing on research activities conducted in Plant Pathology Section; (h) Briefing on Entomology and Nematode Section.

Conclusions

Generally, plant quarantine station in each countries in the world responsible to employ plant quarantine procedures in order to protect their country from introduction of harmful and dangerous pests which potentially affects agricultural industries. Plant Quarantine of Malaysia implements the similar protocols, procedures and regulations for the importation of agricultural products. Malaysian Plant Quarantine authority are strictly implements these procedures especially in protecting our 'golden crop' (oil palm) and rubber from dangerous pests (eg. South American leaf blight, Red palm weevil, etc) which can collapse the palm oil and rubber industries once introduced to the crops. Therefore, cooperation from researches, academician, plant quarantine officers within and outside the country for sharing the information about new pests, identification methods, inspection procedures, biologies of the pests, effective eradication and prevention treatments will helps to prevent the outbreaks and invasion of the pests.

Appendix 16

Other Activities

a) Lectures

Lectures during the attachment. (a) Lecture on plant parasitic nematode by Dr. Marita S. Panili from UPLB, Philippines; (b) Lecture on phylogenetic tree by Dr. Noriko Furuya from DDBJ).

b) International Conferences

International Society for Southeast Asian Agricultural Sciences (ISSAAS)

Halal Seminar

(c) Study Trip, Visit and Festival

NODAI Festival

ISSASS Trip to Kawaguchi Lake and Mount Fuji

Short Attachment at Utsunomiya University

Visit to Yokohama Plant Protection Station main office and Research Centre

Laboratory Visit to Illumina (Next Gene Sequencing) at NODAI

(d) Participants Final Presentation

Attachment 3

Report of Attachment Program
Advanced Diagnostics of Plant Viruses

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan
October 26 – December 25, 2015

By

Nurhayati Madiha
(Malaysia)

Organized by:

Tokyo University of Agriculture
(Tokyo NODAI), Japan
In Collaboration with:

ASEAN Network on Taxonomy

2016

Attachment Program:
Advanced Diagnostic on Plant Viruses

(Taxonomic Capacity Building to Support Market Access for Agricultural Trade in the ASEAN Region)
Tokyo University of Agriculture, Japan

Duration:
26th October 2015 – 25th December 2015

Participant Name &Position:
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1. Background Information

This training ("Training Workshop on the Diagnostics of Plant Viruses") was coordinated by the Institute of Plant Breeding - Crop Science Cluster, College of Agriculture, and the University of the Philippines Los Banos through ASEANET project and Plant Health ASEAN Cooperation Network (APHCN) namely "Taxonomic capacity building to support market access for agricultural trade in the ASEAN region". The project is sponsored by the Japan - ASEAN Integration Fund (JAIF) which will be implemented within two years, includes a number of activities related to trainings and attachment programs.

The project aims to provide a basic understanding of the concepts and practical plant virus, a diagnosis of infected plants, the latest technology and management of diseases involving viral diseases. The topics are as follows: basic knowledge of virus classification, morphology genus of plant viruses, virus transmission, diagnosis is based on symptoms, detection using serological tests (Enzyme-linked immunosorbent assay, ELISA) and molecular techniques (polymerase chain reaction, PCR), virus interests to plant crops in tropical and sub-tropical and virus management methods to prevent the spread of disease.

2. Objectives

The objectives of the attachment program are:

1. To provide diagnostic skills for identification of plant virus disease based on symptoms.
2. To study detection and identification of plant viruses using advanced diagnostic techniques; serological and molecular techniques.
3. To study the transmission of plant viruses from the sources to target hosts.
4. To apply the suitable techniques for plant virus disease management in ASEAN countries.

3. Daily Programs

Date	Programs	Remarks
26th Oct 2015 (Monday)	• Arrived in Tokyo. • Moved into Tokyo NODAI Guest House and visited to the lab. • Short discussion with Prof. Natsuaki on the coming two months attachment schedules.	
27th Oct 2015 (Tuesday)	• All participants were meet up inProf. Hogoken's Laboratory. • Tour around NODAI campus. • Buffer preparation for ELISA test - Phosphate buffer - General Extraction Buffer. - 5X PBST.	The tour was guided by Prof. Natsuaki's students.We have visited the library, Student Coop, administration office and Museum.
28th Oct 2015 (Wednesday)	• Sample collection and symptoms observation on Bamboo virus around NODAI campus. • Detection on Potyvirus from Bamboo samples.	Detection of Potyvirus using ELISA test.
29th Oct 2015 (Thursday)	• Sample collection at the greenhouse (passion fruit, *Passiflora edulis*) • Sap inoculation (back inoculation) of unknown passion fruit virus on healthy passion fruit seedlings. • Sow the 3 types of seeds (passion fruit and beans).	
30th Oct 2015 (Friday)	• Visited to NODAI Museum and Biorium with Prof. Natsuaki. • Attending Tea Ceremony during Festival.	
31st Oct 2015 (Saturday)	• Attending NODAI Festival and welcome party with Professor and students from Laboratory of Tropical Plant Protection.	

2nd Nov 2015 (Monday)	• Preparation of negative stain reagent (PTA buffer). • Sample preparation for electron microscope observation to detect plant virus particles.	
3rd Nov 2015 (Tuesday)	-	National holiday
4th Nov 2015 (Wednesday)	• Detection of Potyvirus from passion fruit using Indirect ELISA technique.	
5th Nov 2015 (Thursday)	• Detection of Potyvirus from taro using Indirect ELISA technique.	
7th–9thNov 2015 (Sat– Mon)	• ISSAAS Conference and trip to Mount Fuji.	
10th Nov 2015 (Tuesday)	-	Off day
11th Nov 2015 (Wednesday)	• Briefing and orientation • DNA extraction from BBTV-infected banana. • PCR and gel electrophoresis.	
12th Nov 2015 (Thursday)	• Pre-lab discussion • Short topic presentation on plant parasitic nematodes. • Gel electrophoresis. • Extraction and detection of BBTV from fresh and old banana sample and abaca samples.	
13th Nov 2015 (Friday)	Post-lab discussion	
16th Nov 2015 (Monday)	• Gel electrophoresis of BBTV PCR products. • Impregnation and extraction of virus nucleic acid from FTA plant card.	
17th Nov 2015 (Tuesday)	• PCR assay of DNA from FTA plant card. • Gel electrophoresis. • Gel cut, purification of DNA products.	
18th Nov 2015 (Wednesday)	• Extraction of BBTV directly from insect vector, *Pentalonia nigronervosa*. • PCR assay.	

	• Ligation of purified DNA using pGEM vector.	
19th Nov 2015 (Thursday)	• Extraction of BBTV DNA from aphids impregnated on FTA card. • PCR assay. • Gel electrophoresis. • Transformation of ligated plasmid.	
20th Nov 2015 (Friday)	• Checking of colonies/transformants. • Post-lab discussion.	
23rd Nov 2015 (Monday)	• Picking of transformed colonies. • Culturing of *E. coli* colonies into LB medium.	
24th Nov 2015 (Tuesday)	• Mini-prep. • Insert check. • Visit to electron microscope laboratory.	
25th Nov 2015 (Wednesday)	• Precipitation. • DNA sequencing.	
26th Nov 2015	• Special lecture. • Sequence analysis.	Lecture on bioanalysis and phylogenetic tree by Dr. Noriko Furuya from DDBJ.
27th Nov 2015 (Friday)	Post-lab discussion.	
30th Nov 2015 (Monday)	• Discussion with Sensei about the Yokohama Trip and December schedules. • Halal Seminar	
1st Dec 2015 (Tuesday)	• Analyze the sequencing results using Bioseq software, BLAST.	
2ndDec 2015 (Wednesday)	• Preparation of LB Medium	
3rd Dec 2015 (Thursday)	• Attachment at Utsunomiya University on dsRNA Extraction of Cucumber Mosaic Virus (CMV).	Under supervision by Prof.Tomohide Natsuaki.
4th Dec 2015 (Friday)	Post-lab discussion	
7th Dec 2015 (Monday)	• Potyvirus RNA Extraction from passion fruit	
8th Dec 2015 (Tuesday)	• cDNA synthesis	

9th Dec 2015 (Wednesday)	• Gel electrophoresis of Potyvirus cDNA	
10th Dec 2015 (Thursday)	• cDNA synthesis from Potyvirus (repeat). • Preparation of chemical (chloride for TB) and TB medium.	cDNA synthesis was repeated for second time because we could not get the good quality of cDNA product.
11th Dec 2015 (Friday)	• Visit to Yokohama Plant Protection Section and Research Centre.	
14th Dec 2015 (Monday)	• Gel electrophoresis • Gel Extraction (Purification of cDNA)	
15th Dec 2015 (Tuesday)	• Ligation • SDS-Page Protocol • Western Blot Protocol • Preparation of reports and final presentation.	SDS-Page and Western Blot laboratory protocol was conducted by Chong Sensei.
15th Dec 2015 (Wednesday)	• Transformation of *E. coli*	National holiday
16th Dec 2015 (Thursday)	• Checking the *E. coli* colonies. • Culturing of *E. coli* on TB Medium (Repeat 2nd time).	The colonies obtained on TB medium is not enough to be used for Mini Prep and sequencing. Therefore, 3rd time ligation was done to get better result.
17th Dec 2015 (Friday)	• Purification of dsRNA • Attending PhD Thesis Defends from Ayaka Uke.	
21st Dec 2015 (Monday)	• Final presentation for 3 ASEAN participants on attachment program. • Ligation (3rd time)	
22nd Dec 2015 (Tuesday)	• Transformation (3rd time) • Finishing all reports and submit to Sensei.	
23rd Dec 2015 (Wednesday)	• Checking the *E. coli* colonies. • Culturing *E. coli* in TB medium (3rd time).	
24th Dec 2015 (Thursday)	• Mini Prep. • Move out from NODAI Guest House and went to Narita Hotel.	
25th Dec 2015 (Friday)	• Going back to Malaysia.	

Saturday and Sunday – Free day

4. Activities during Attachment Program

(a) Laboratory Studies

(i) Buffer Preparation for ELISA Test

We were assisted by the Hogoken's students for the preparation of phosphate buffer, general extraction buffer, and 5X PBST for ELISA test. We also learned the preparation culture medium for the *E. coli* (TB and LB medium). These two types of media will be used for *E. coli* culturing and cloning procedure. All ingredients and preparation protocols for buffers are shown in Appendix 1 – 2.

(ii) Symptoms Observation and Sample Collection (Refer to Appendix 3 – 4)

Several types of plants were collected from NODAI and outside the university campus for detection of the virus infection. First sample which was collected around the NODAI is bamboo plant (*Pleioblastus chino)*. The bamboo leaves were suspected to be infected by Potyvirus. The symptoms were showed streaking along the leaves and yellow mosaic pattern compare with healthy bamboo leaves. These symptoms are similar to the sugarcane viruses.By using ELISA method, it will help to differentiate the viruses.

Second sample was taro leaves. The samples were taken by one of the student from other prefecture and from the growth chamber at Hogoken's laboratory. The leaves were showed mosaic pattern on the leaves. The samples were subjected for Potyvirus detection using ELISA test

Third sample was passion fruit. The leaves were taken from glasshouse at NODAI. The leaves also used for sap inoculation on healthy passion fruit leaves. The symptoms were not obvious.The leaves were used for ELISA test for detection of Potyvirus.

Banana leaves which were infected with Banana bunchy top virus (BBTV) were taken inform the growth chamber at Hogoken's laboratory. The plants were highly infested with the aphid (*Pentalonia negronervosa*). Only

leaveswere used for the detection and identification of BBTV using molecular technique and FTA cards. For the aphids, sample was taken from the same infested plants and subjected for FTA cards impregnation and molecular identification.

(iii) Detection of Plant Viruses using Indirect ELISA

Samples taken (bamboo, taro and passion fruit) were subjected for the detection of Potyvirus using Indirect ELISA. The ELISA procedures were shown in Appendix 5 – 7.

(iv) Identification of Plant Viruses using Molecular Techniques (Extraction of DNA, RNA and dsRNA, Polymerase Chain Reaction (PCR), Reverse Transcriptase (RT) PCR, Gel electrophoresis, Purification of PCR Product, Cloning and Sequencing)

Samples from banana infected with BBTV, aphids and Cucurbit leaves (Cucumber mosaic virus) were used for identification of virus using all the techniques mentioned above. The samples were extracted from fresh infected leaves, fresh aphids and samples impregnation on FTA cards. Full procedures were shown in Appendix 8 – 12.

(v) SDS-PAGE and Western Blot

SDS-PAGE and western blot is one of the method can be used for detection of virus protein instead of using ELISA and molecular techniques by separating the protein mixture according to their molecular weight. This training was conducted by Mr. Chong from NODAI. Details protocol was shown in Appendix 13.

(b) Field Study/ Visit

(i) Visit to NODAI's Museum and Biorium

The visit was done with Prof Keiko T. Natsuaki. During the visit, we get to know about the history of Tokyo University of Agriculture and Japanese tradition.

(ii) **Visit to Utsunomiya University**

The visit was done in early of December for 2 days. This attachment was supervised by Prof. Tomohide Natsuaki and his students. During the 2 days attachment, we learned about dsRNA extraction for detection of dsRNA of Cucumber mosaic virus (CMV) from Cucurbit plant. Protocol details were shoen in Appendix 14.

(iii) **Visit to Yokohama Plant Protection Station**

The visit was started at the main office of Yokohama Plant Protection Station situated in Yokohama. During the visit, the plant quarantine officer giving a tutorialon the Japan's Plant Quarantine Policies, regulations and implementation of Plant Quarantine Act for all the importation and exportation items, electronic system for application of import permit and others.

In the afternoon, we moved to the Research Centre of Yokohama Plant Quarantine Station. In this Research Centre, we have been shown on their roles to conduct researchers on pests, diseases, pest risk analysis and plant quarantine treatments. The research conducted by this centre helps to provide beneficial information for the application of plant quarantine regulation in Japan.Refer to Appendix 15 for full report.

(c) Program of Conferences

(i) **International Society for Southeast Asian Agricultural Sciences (ISSAAS) – International Congress**

The congress was held on 7 – 9 November at NODAI. From the congress, we gained more knowledge from all the lectures presented by ASEAN and Japanese participants from quarantine agencies and universities. I have a good chance to attend their presentation regarding to the research on pests and diseases, biocontrol of plant pathogens, nutrient application on oil palm, diversity of plant viruses and current methodology in identification of pathogens such as detection of plant viruses using immunochromatographic strip developed by researcher from Thailand. Besides that I met colleagues from Malaysia and other countries. We were able to share knowledge and opinions together during the congress.

On the final day of the congress, we participated in the field trip to Kawaguchi Lake and Mount Fuji.

(ii) **Halal Seminar**

This seminar was held on 30 November in NODAI. I was attended one of the lecture presented by Dr. Dzulkifly Mat Hashim from Universiti Putra Malaysia on Halal Food Authentication in Malaysia. The purpose of this seminar is to introduce the halal industry, halal certificates, technology for halal test and others. This is important for Malaysia to introduce the halal concept to the Japanese and increase their understanding on the application of halal for the Muslim and food industry and become one of the attraction for tourists to visit Japan.

5. Summary and Recommendations

In general, the attachment program has reached the main objectives to provide capacity building for the ASEAN countries to learn and gain knowledge specifically on diagnostic of plant viruses. The training was conducted and supervised by expert resource persons on plant viruses and as a chosen participant, I have good experiences in order to learn the basic and advance knowledge in plant viruses diagnostic. I was able to create a networking and sharing some ideas with Japan's agencies, professors and students and build cooperation between ASEAN countries and Japan in plant quarantine enforcement. With the relationship built up, it may help to improve our diagnostic skills in plant quarantine test procedures starting from symptoms observation until identification of the plant viruses for agriculture products trade, understanding the importance of plant viruses and applying the correct management techniques in order to control the spread of plant virus infections within the country.

In conclusion, this program provided me a good exposure to new technology and improved my technical skills in detection and identification of plant viruses. I will applied and share the skills and techniques learned during the attachment program to my colleagues in my department. In addition, my recommendation for next training is to conduct training program on plant parasitic nematodes. The limitation of expertise in ASEAN countries especially for identification of plant parasitic nematodes is a constraint for us in managing the nematodes infection. Such training will helps us to increase our understanding and technical skills for early detection of disease infestation, species identification and suitable control measures to prevent the infection by plant parasitic nematodes.

6. Acknowledgement

I would like to express my deepest appreciation to the sponsor of this project, Japan-ASEAN Integration Fund (JAIF) for giving me the opportunity to ASEAN countries to involve in this capacity building program.

I place on record my sincere gratitude to Dr.Lum Keng Yeang and Dr. Soetikno Selamat for giving me chance to be selected for the attachment program in Japan. Grateful acknowledgement to Prof. Keiko T. Natsuaki sensei for her time and effort, guidance, sharing her experiences, taking care our needs and accommodate us with comfortable accommodations and giving us advices.

I would like to thank Dr. Marita S. Panili, Prof. Tomohide Natsuaki, Mr. Chong sensei and all the Hogoken's students for sharing the valuable knowledges, experiences, encouragement and guidance during our short attachment with them. Thank you very much for everything.

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Attachment 4

Report of Attachment Program
Advanced Diagnostics of Plant Viruses

at
Laboratory of Tropical Plant Protection
Tokyo University of Agriculture (Tokyo NODAI), Japan
October 26 – December 25, 2015

By

Tran Van Chien
(Viet Nam)

Organized by:

Tokyo University of Agriculture
(Tokyo NODAI), Japan

In Collaboration with:

ASEAN Network on Taxonomy

2016

REPORT OF ATTACHMENT PROGRAM IN JAPAN

FROM October 27th to December 24th 2015

Prepared by M.Sc. **Tran Van Chien**

Plant Quarantine Officer

Post-Entry Quarantine Center No.1

Plant Protection Department - Ministry of Agriculture & Rural Development

Country: Vietnam

1. Background information

The ASEAN Plant Health Cooperation Network (APHCN) – ASEANET Project ***"Taxonomic capacity building to support market access for agricultural trade in the ASEAN region"***, funded by the Japan ASEAN Integration Fund (JAIF) has been implemented for 2 (two) years starting from 15th May 2015 covering several activities related to training and attachment program.

Due to the first workshop ***"Training Workshop on Plant Viruses"***, which was successfully held from 17-28 August 2015 at UPLB, Philippines with nine ASEAN countries including Brunei Darussalam, Indonesia, Cambodia, Laos, Malaysia, Myanmar, Philippine, Thailand, and Vietnam. Under this ASEAN-endorsed Project, three outstanding participants from the training workshop are to be selected for a two-month attachment program "***Training Workshop on Advanced Diagnostics on Plant Viruses"*** at the Laboratory of Tropical Plant Protection of the Tokyo University of Agriculture (TUA), Japan, for further training under supervision of Prof. Keiko Natsuaki (Vice President of TUA) and Dr. Marita S. Pinili (Institute of Plant Breeding, University of the Philippines Los Banos).

2. Objectives of the attachment program

- To provide diagnostic skills for identification of plant virus diseases based on typical symptoms.
- To study the methods for detection and identification of plant viruses by serological and molecular techniques.
- To study the transmission of plant viruses from the sources to target host.
- To apply the suitable techniques for plant viruses disease management in our country.

3. Daily program

Date	Activity	Venue	Resource Person(s)
Monday Oct. 26th	Arriving at Tokyo University of Agriculture (TUA) and visit Laboratory of Tropical Plant Protection (HOGOKEN Lab.)	Tokyo University of Agriculture (TUA) and HOGOKEN Lab.	Mr. Takeda
Tuesday Oct. 27th	Visiting the campus and making phosphate buffer for mechanical inoculation.	HOGOKEN Lab.	Mr. Chung; Ms Kudo and Mr. Ikeda
Wednesday Oct. 28th	Detecting virus on bamboo by ELISA method	HOGOKEN Lab.	Ms. Hiraiwa and Ms Matsumoto
Thursday Oct. 29th	- Inoculating *potyvirus* to passionfruit seedlings by mechanical method and sawing the index plants. - Visiting the NODAI Biorium	HOGOKEN Lab.	Prof. Keiko Natsuaki and Mr. Ikeda
Friday Oct. 30th	- Sampling the samples with virus-like symptom. - Wrapping up of the first week.	HOGOKEN Lab.	Ms. Hiraiwa and Ms Takada
Saturday Oct. 31st	Attending the TUA's festival and the dinner party at the HOGOKEN Lab.		
Sunday Nov. 1st	Day off		
Monday Nov. 2nd	Preparing the sample conservation buffers	HOGOKEN Lab.	Ms. Hiraiwa and Mr. Ikeda
Tuesday Nov. 3rd	Day off – national holiday		
Wednesday Nov. 4th	Detecting virus on passionfruit by ELISA method	HOGOKEN Lab.	Mr. Ikeda
Thursday Nov. 5th	Detecting virus on passionfruit by ELISA method (continued)	HOGOKEN Lab.	Ms. Takada
Friday Nov. 6th	- Using the information network regarding plant virus identification (DDBJ/NCBI).	HOGOKEN Lab.	Prof. Keiko Natsuaki

Date	Activity	Venue	Resource Person(s)
	- Wrapping up of the second week.		
Saturday Nov. 7th	Attending the ISSAAS conference.	NODAI Academia Center	
Sunday Nov. 8th	Attending the ISSAAS conference.	NODAI Academia Center	
Monday Nov. 9th	Attending the ISSAAS excursion.	Kawaguchiko Lake; Fuji mountain	
Tuesday Nov. 10th	Day off – catch up holiday for Nov. 8th		
Wednesday Nov. 11th	- DNA extraction from *Banana bunchy top virus* (BBTV)-infected banana samples. - Running PCR reaction and preparing the agarose gel.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 12th	- Short presentation on plant parasitic nematodes. - Gel electrophoresis. - Extraction and detection of BBTV from fresh and preserved banana and abaca samples.	HOGOKEN Lab.	Dr. Marita S. Pinili
Friday Nov. 13th	Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 14th	Day off		
Sunday Nov. 15th	Day off		
Monday Nov. 16th	- Conducting gel electrophoresis of BBTV PCR products. - Impregnation and extraction of virus nucleic acid from FTA plant card.	HOGOKEN Lab.	Dr. Marita S. Pinili
Tuesday Nov. 17th	- Performing PCR assay of DNA from FTA plant card.	HOGOKEN Lab.	Dr. Marita S. Pinili

Date	Activity	Venue	Resource Person(s)
	- Carrying out gel electrophoresis and gel cut. - Purification of DNA product.		
Wednesday Nov. 18th	- Extraction of BBTV from banana aphids (*Pentalonia nigronervosa)* as a vector of virus. - Conducting the PCR assay. - Ligation of purified DNA using pGEM vector.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 19th	- Extraction of BBTV from aphid impregnated on FTA plant card. - Conducting the PCR assay and gel electrophoresis. - Transformation of ligated plasmid.	HOGOKEN Lab.	Dr. Marita S. Pinili
Friday Nov. 20th	- Checking the colonies of transformation step. -Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 21st	Day off		
Sunday Nov. 22nd	Day off		
Monday Nov. 23rd	- Picking the transformed colonies. - Culturing the *E.coli* colonies to LB medium.	HOGOKEN Lab.	Dr. Marita S. Pinili
Tuesday Nov. 24th	- Performing miniprep and insert check.	HOGOKEN Lab.	Dr. Marita S. Pinili
Wednesday Nov. 25th	- Conducting the precipitation step. - Preparing for DNA sequencing.	HOGOKEN Lab.	Dr. Marita S. Pinili
Thursday Nov. 26th	- Attending the lecture on Phylogenetic tree and	HOGOKEN Lab.	Dr. Noriko Furuya (from DNA Data Bank

Date	Activity	Venue	Resource Person(s)
	constructing phylogenetic tree by MEGA software.		of Japan – DDBJ).
Friday Nov. 27th	-Post-laboratory discussion	HOGOKEN Lab.	Dr. Marita S. Pinili
Saturday Nov. 28th	Day off		
Sunday Nov. 29th	Day off		
Monday Nov. 30th	- Discussion about the plan schedule of Yokohama Trip and short visit to Utsunomiya University. - Attend Halal Seminar by Nodai cooperate with Putra Malaysia University	HOGOKEN Lab. NODAI Academia Center	Prof. Keiko Natsuaki
Tuesday Dec. 1st	Preparation of LB medium for culturing bacteria	HOGOKEN Lab.	Ms. Takada
Wednesday Dec. 2nd	Transferring the banana aphids from healthy plant to BBTV-infected plant for acquisition of virus for transmission experiment.	HOGOKEN Lab.	Ms. Takada
Thursday Dec. 3rd	Visiting to Utsunomiya University to learn the method for dsRNA extraction.	Laboratory of Plant Pathology	Dr. Tomohide Natsuaki and his students
Friday Dec. 4th	Continue dsRNA extraction.	Laboratory of Plant Pathology	Dr. Tomohide Natsuaki and his students
Saturday Dec. 5th	Day off		
Sunday Dec. 6th	Day off		
Monday Dec. 7th	- Conducting RNA extraction for potyvirus from passion fruit (inoculated plant) using phenol chloroform method.	HOGOKEN Lab.	Ms. Minho

Date	Activity	Venue	Resource Person(s)
	- Performing the cDNA synthesis assay.		
Tuesday Dec. 8th	Carrying out the PCR assay and gel electrophoresis.	HOGOKEN Lab.	Mr. Ikeda
Wednesday Dec. 9th	Gel checking	HOGOKEN Lab.	Ms. Matsumoto
Thursday Dec. 10th	- Re-conducting the PCR assay because of not good result. - Preparing the LB medium	HOGOKEN Lab.	Ms. Takada
Friday Dec. 11th	- Study visit to Yokohama Plant Protection Station and Research Center.		Prof. Keiko Natsuaki
Saturday Dec. 12th	Day off		
Sunday Dec. 13th	Day off		
Monday Dec. 14th	Performing the gel electrophoresis and DNA purification.	HOGOKEN Lab.	
Tuesday Dec. 15th	- Performing the DNA ligation. - Detection of protein using SDS-PAGE and western blot	HOGOKEN Lab.	
Wednesday Dec. 16th	Performing the DNA transformation.	HOGOKEN Lab.	
Thursday Dec. 17th	Conducting dsRNA purification.	HOGOKEN Lab.	
Friday Dec. 18th	Attending the doctoral thesis defense of Ms. Ayaka Uke	HOGOKEN Lab.	
Saturday Dec. 19th	Day off		
Sunday Dec. 20th	Day off		
Monday Dec. 21st	- Performing the final presentation for attachment project.	HOGOKEN Lab.	

Date	Activity	Venue	Resource Person(s)
	- Conducting the DNA ligation step.		
Tuesday Dec. 22nd	- Conducting the DNA transformation step. - Visiting Bio-molecular laboratory to gain the information about illumina's sequencing technology		
Wednesday Dec. 23rd	Culturing *E.coli* in TB medium	HOGOKEN Lab.	
Thursday Dec. 24th	- Conducting the miniprep step. - Moving to Narita view Hotel	HOGOKEN Lab.	
Friday Dec. 25th	- Coming back to Vietnam		

4. Activities

4.1. Laboratory studies:

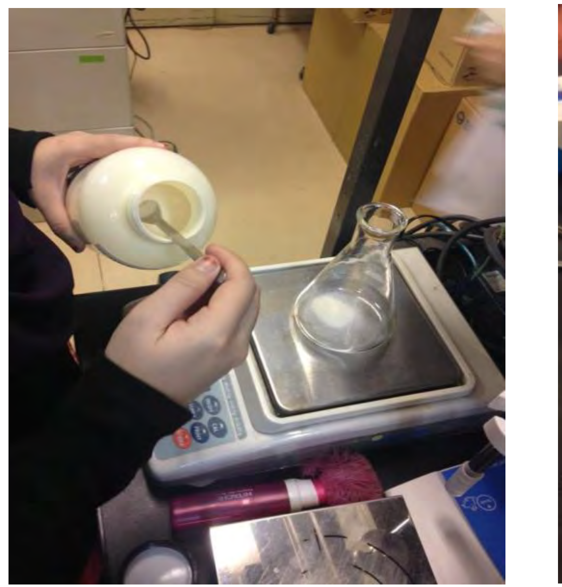
After two months in Tokyo University of Agriculture (TUA), I have gained a lot of valuable knowledge related to detection and identification of plant virus diseases.

4.1.1. Observation of symptoms of samples affected by plant viruses

Fig 1. Observation and samples collecting with virus infection symptoms

Fig. 2: Symptoms of virus infection on Bamboo (*Pleioblastus chino*); Passion fruit (*Passiflora edulis*); Taro (*Colocasia esculenta*) and Banana (*Musa* sp.)

4.1.2. Preparation of several buffers for diagnosis of plant viruses



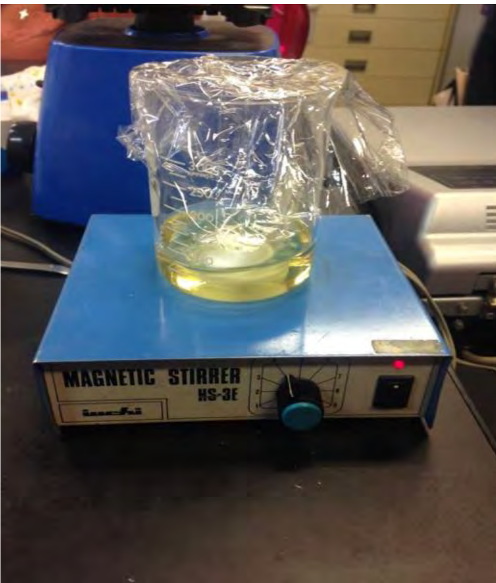


Fig. 3: Making buffers for ELISA technique

4.1.3. Detection of plant viruses using ELISA method

During the program, ELISA method was used for detection of potyvirus on Bamboo (*Pleioblastus chino*), Passion fruit (*Passiflora edulis*) and Taro (*Colocasia esculenta*).

Fig. 4: Detection of potyvirus from selected crops using for ELISA technique

4.1.4. Mechanical inoculation of potyvirus onto Passion fruit seedlings

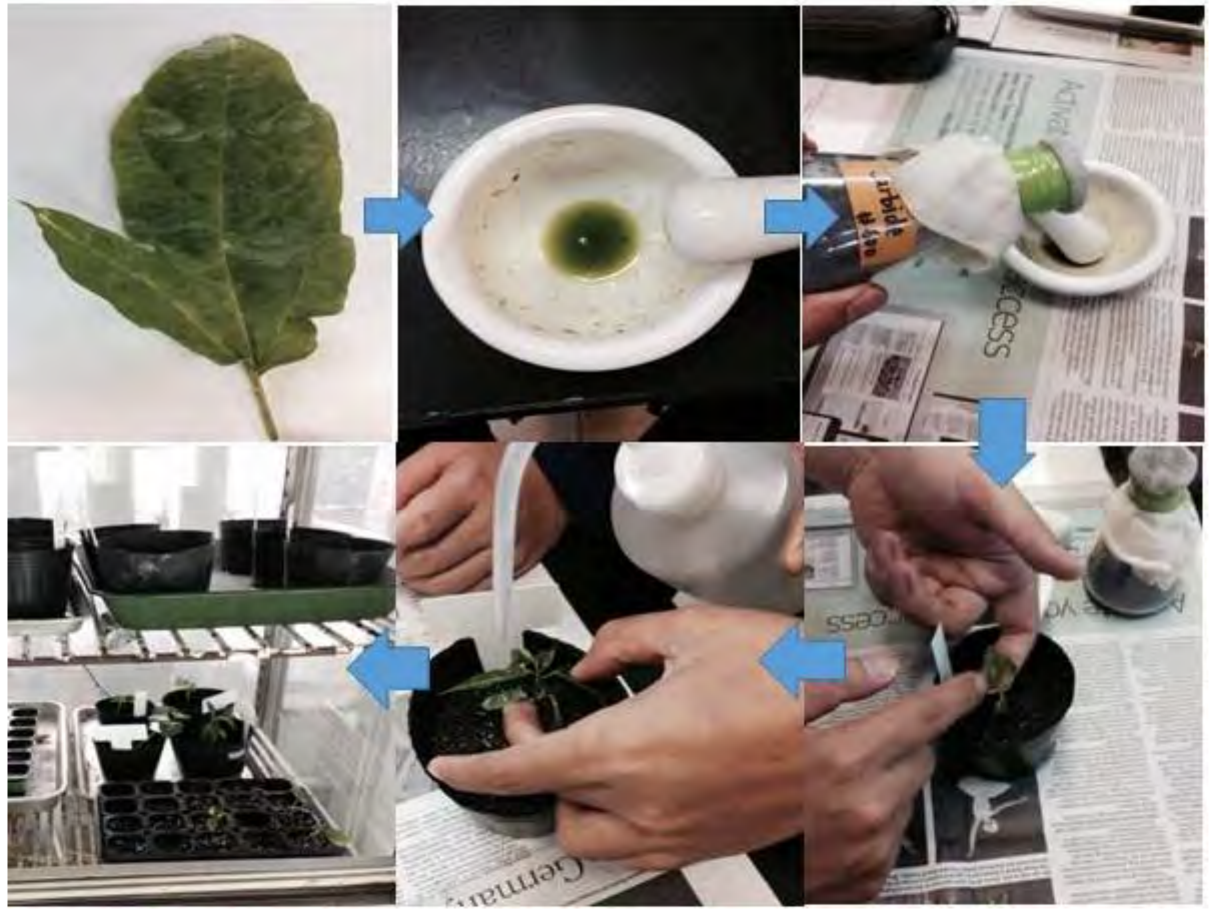


Fig. 5: The process of mechanical inoculation of potyvirus onto Passionfruit seedlings

4.1.5. Detection of Banana Bunchy Top Virus (BBTV) on Banana and Abaca samples using PCR technique

Fig. 6: Preparation for leaf extraction from selelcted samples

Fig. 7: Performance of PCR assay

Fig. 8: Performance of gel electrophoresis

Fig. 9: Gel staining with EtBr and viewing of DNA band under UV transilluminator

4.1.6. Detection of plant viruses using Electron Microscope

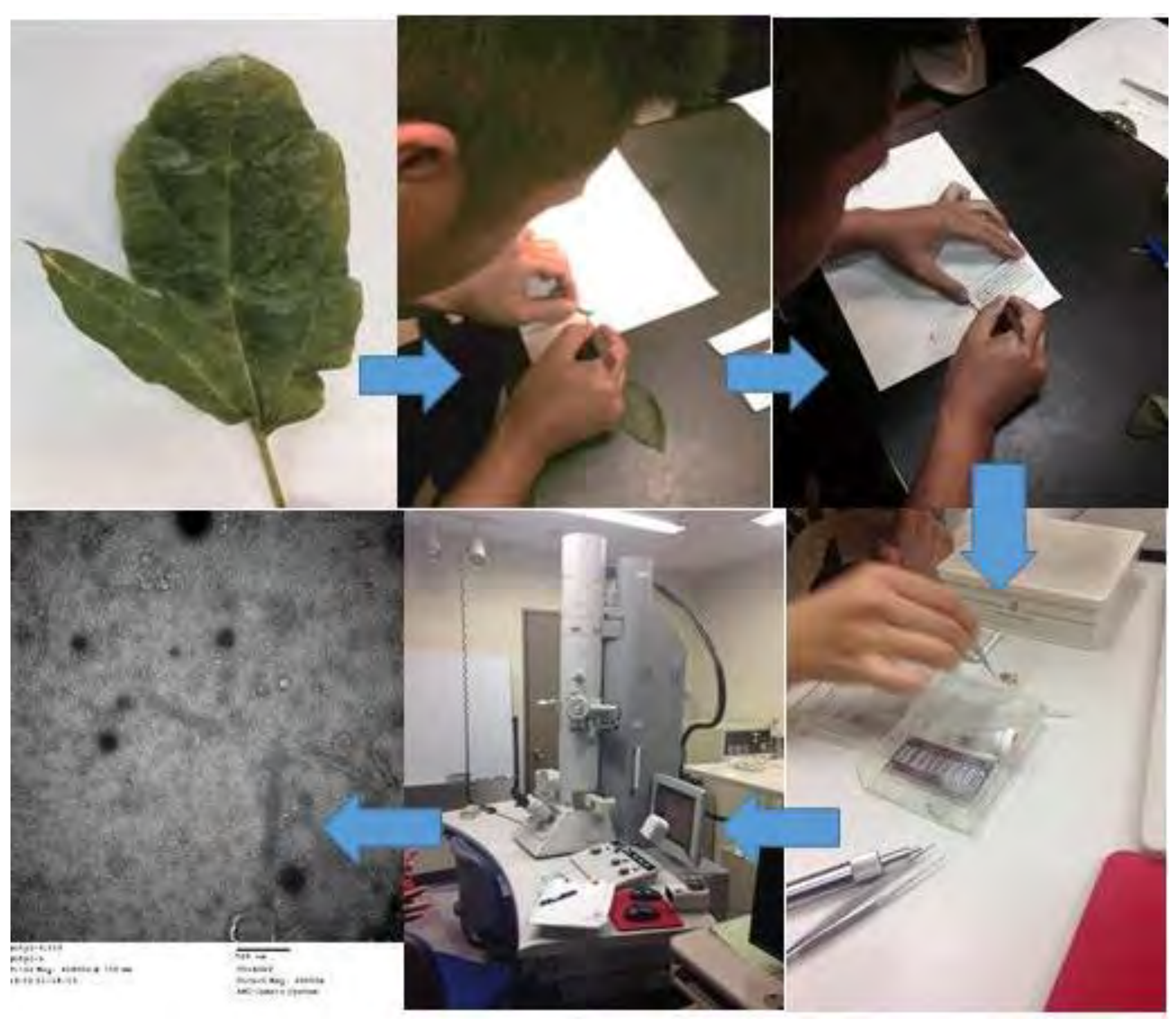


Fig. 10: The process of detection of plant viruses using Electron Microscope

4.1.7. Preservation of virus-infected samples and aphids on FTA plant card

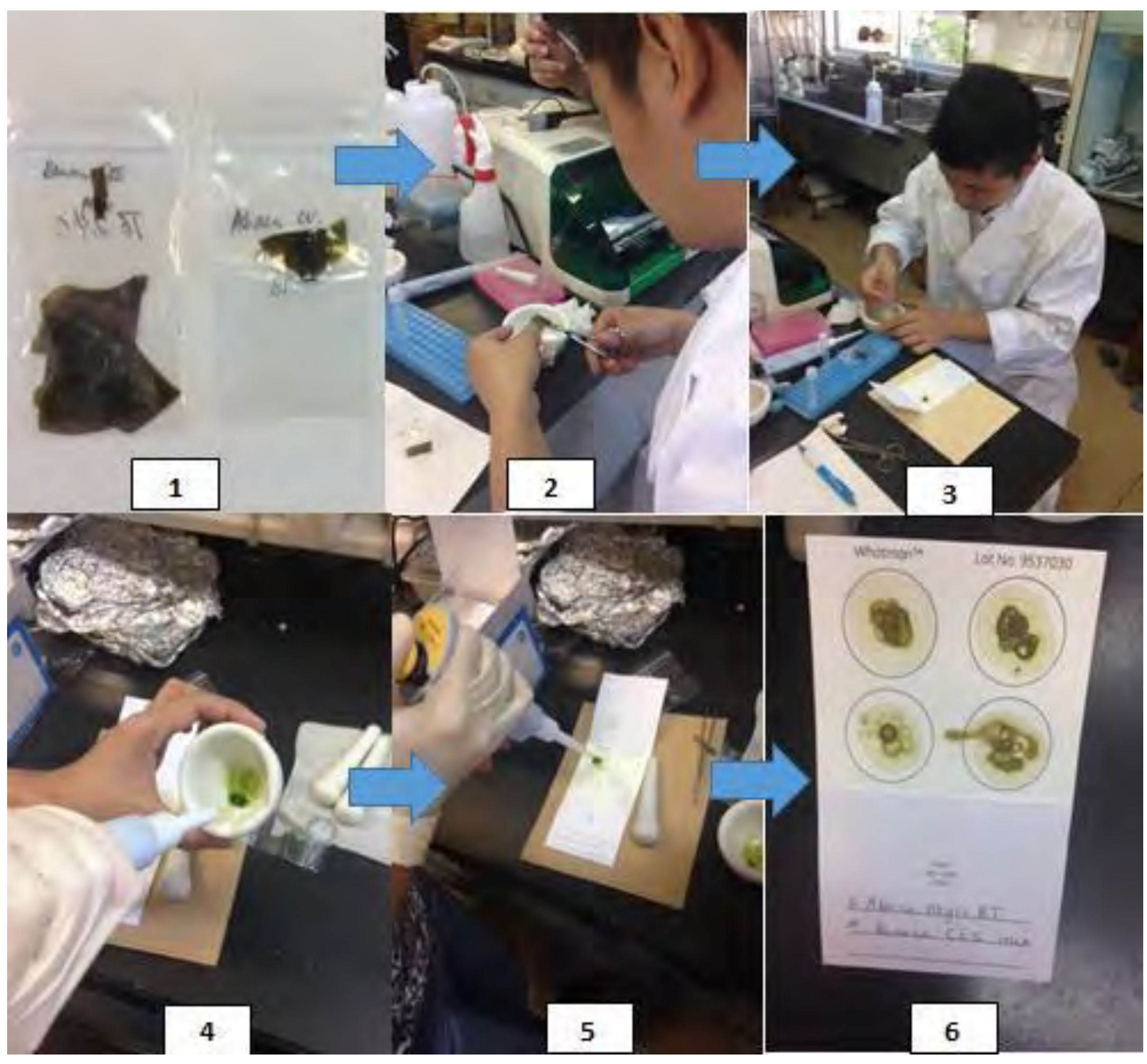


Fig. 11: Preservation of virus-infected leaf on FTA plant card

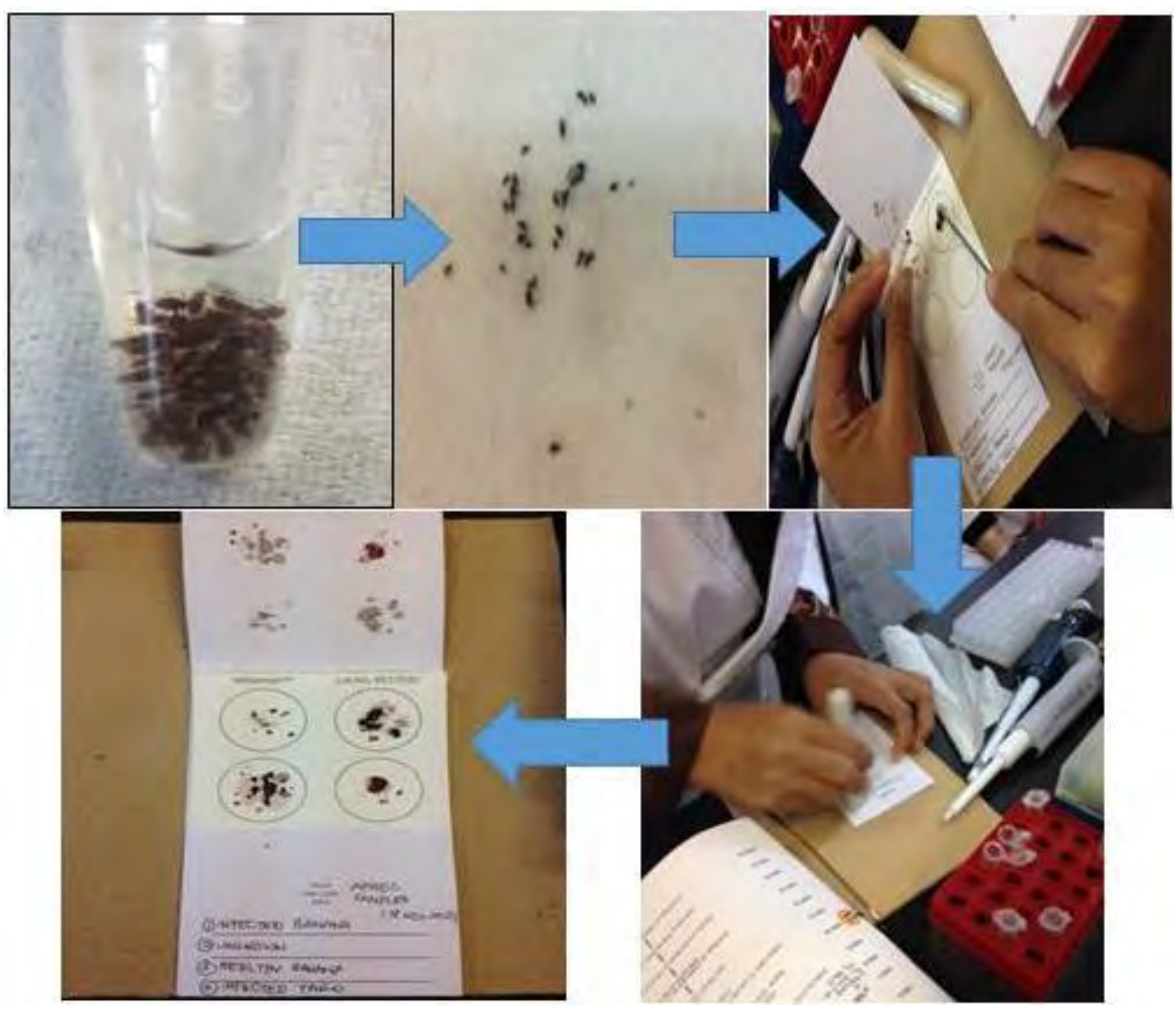


Fig. 12: Preservation of aphids (vector of plant viruses) on FTA plant card

4.1.8. Detection of plant viruses from samples impregnated on FTA plant card

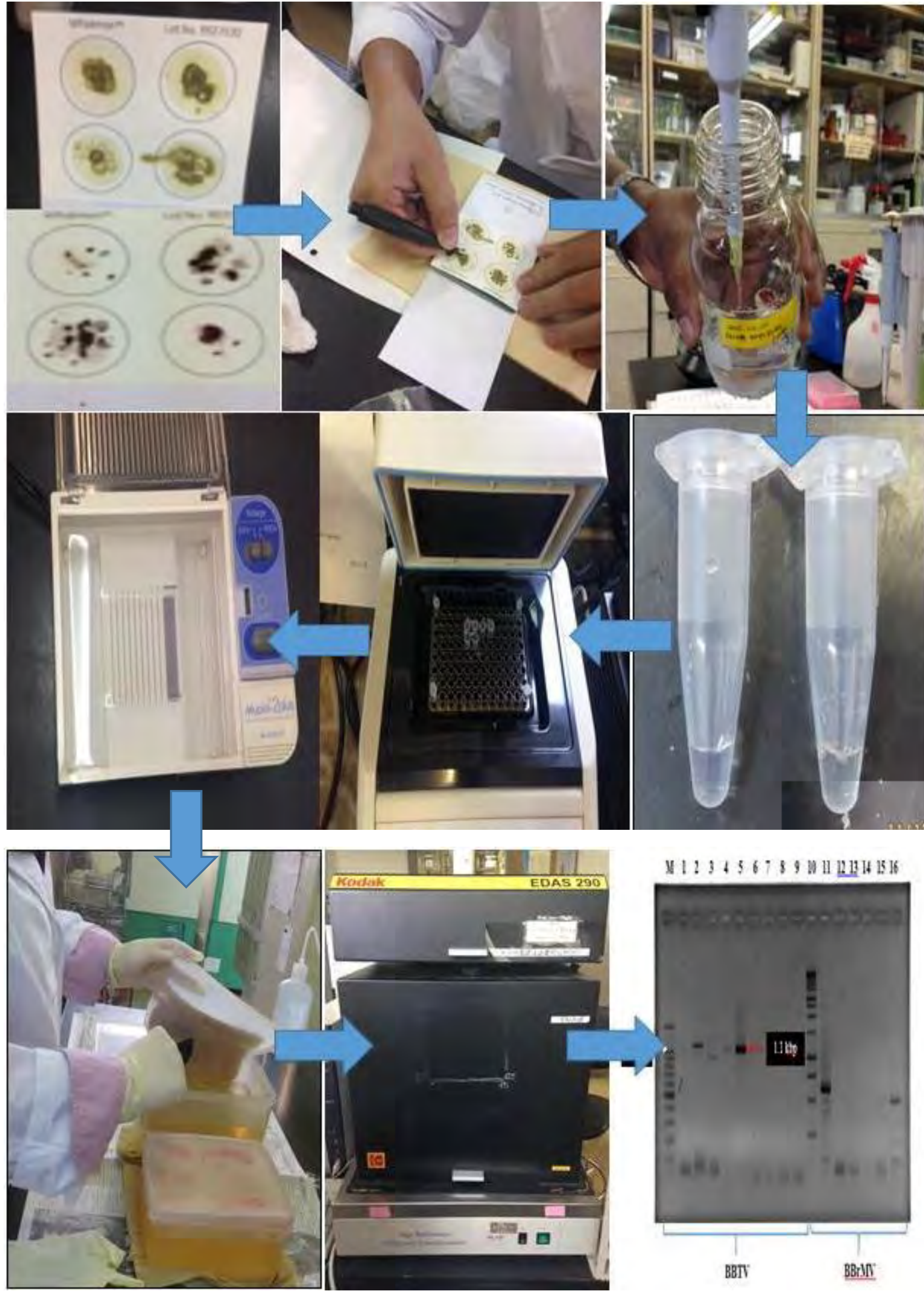


Fig. 13: Detection of plant viruses from samples impregnated on FTA plant card

4.1.9. Method for DNA purification

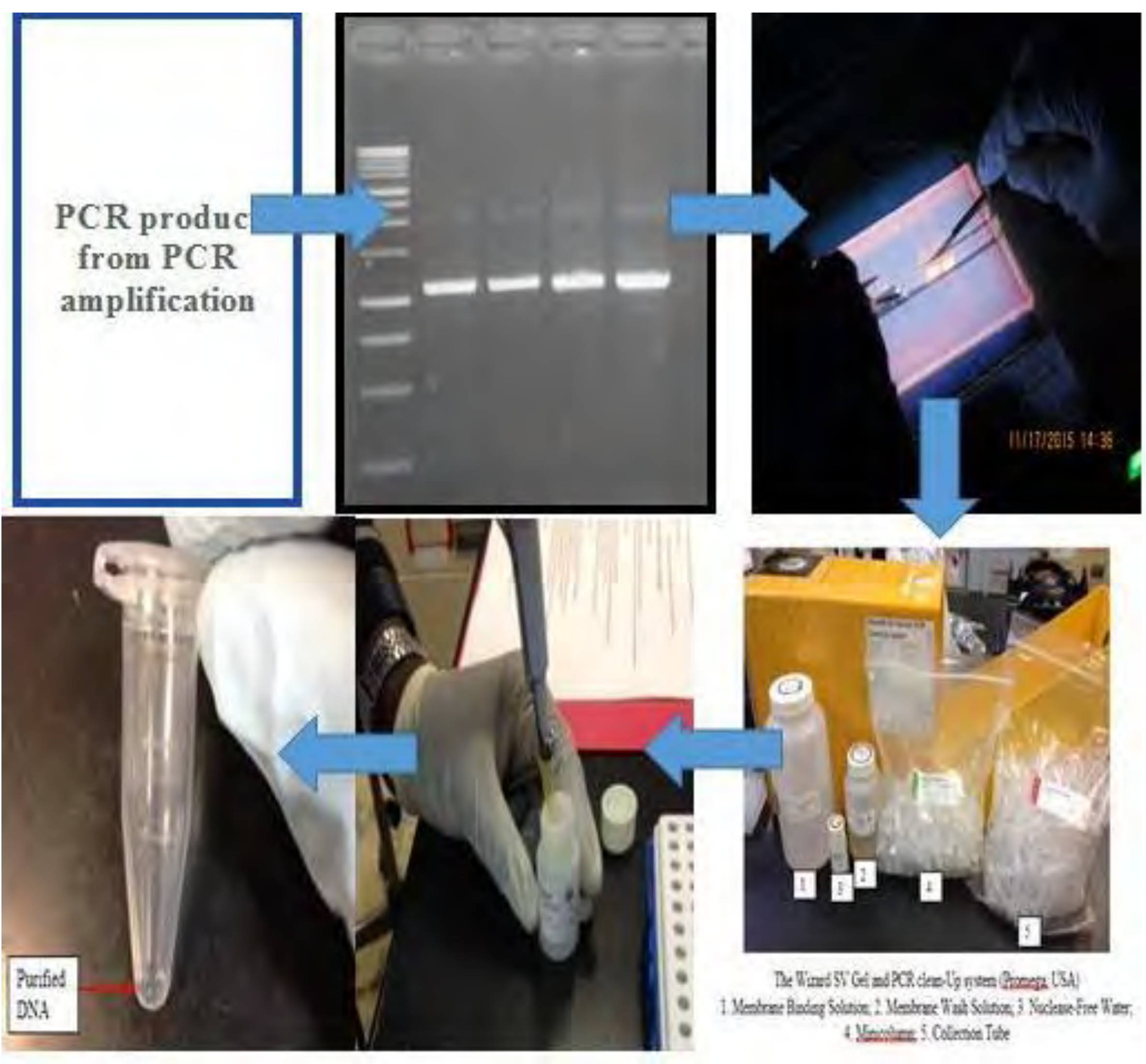


Fig. 14: DNA purification from gel agarose

4.1.10. Method for cloning

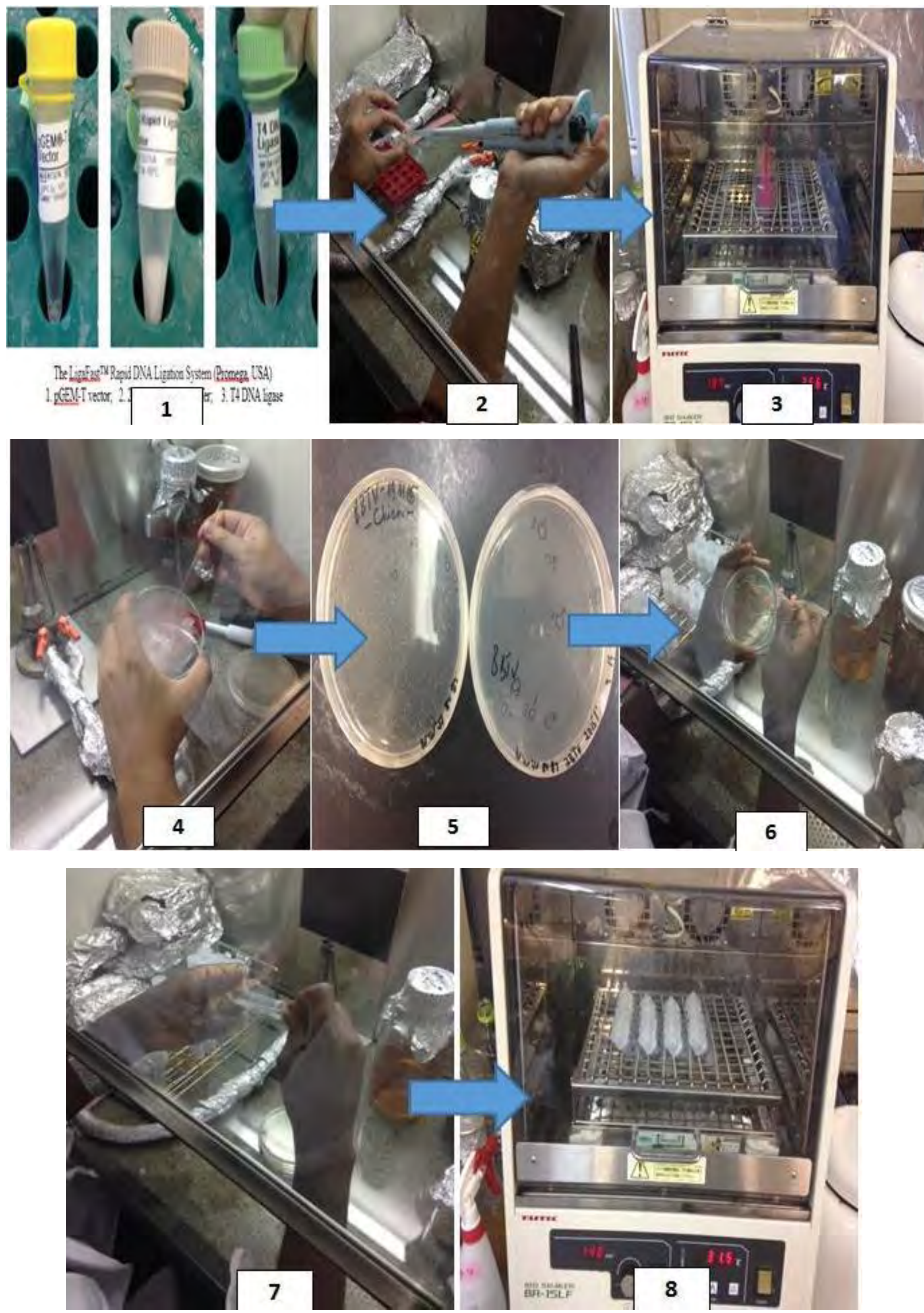


Fig. 15: Cloning of pGEM-inserted colonies

4.1.11. Method for DNA Sequencing

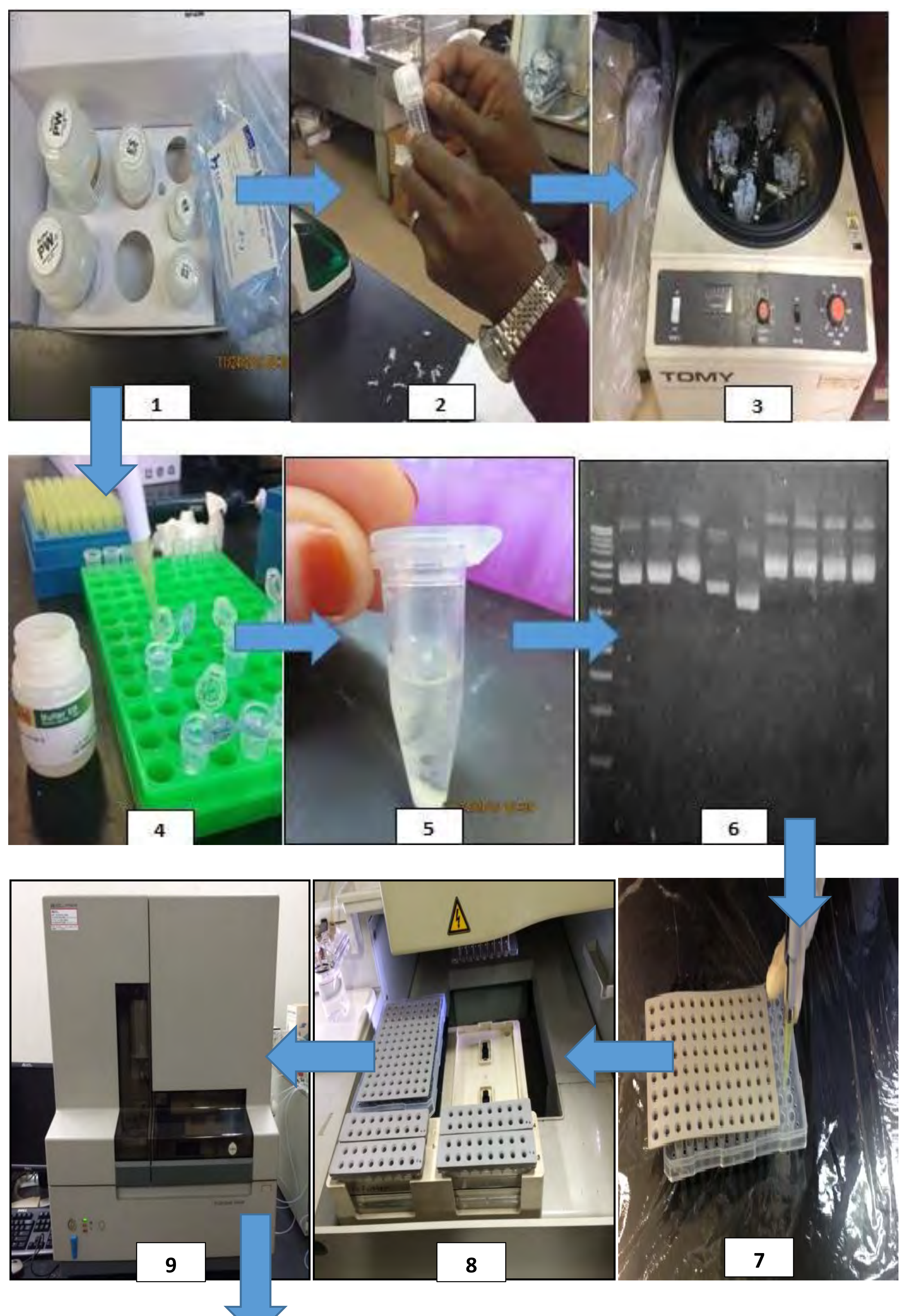


Fig. 16: DNA sequencing of virus isolated from virus infected plant

4.1.12. *Construction of phylogenetic tree using MEGA software*

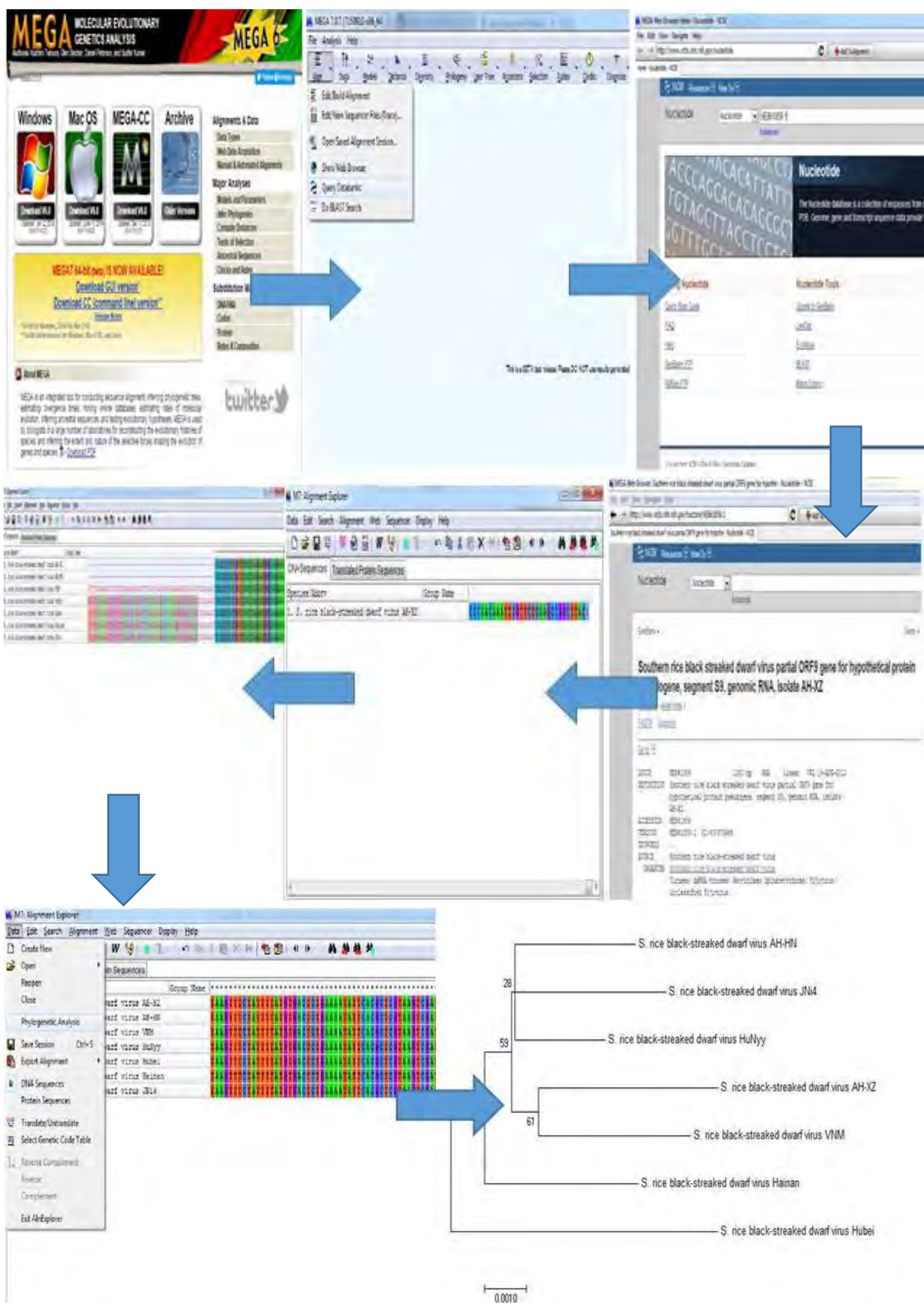


Fig. 17: Construction of phylogenetic tree using MEGA software

4.1.13. Extraction of double-stranded RNA(dsRNA) of plant to detect CMV

Fig. 18: Extraction of dsRNA plant to detect CMV

4.1.14. RNA extraction of plant viruses using phenol-chloroform method

Fig. 19: Extraction of RNA of plant viruses using phenol-chloroform method

4.1.15. Detection of protein present using SDS PAGE (Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis) & Western Blot

Fig. 20: Pictures show SDS PAGE method and Western Blot for detection of protein from bovine serum albumin (BSA)

Fig 21: Attending the lecture on plant parasitic nematode as vector of plant viruses by Dr. Marita S. Pinili

Fig 22: Attending the lecture on DNA sequencing analysis and phylogenetic tree by Dr. Noriko Furuya from DNA Data Bank of Japan (DDBJ)

Fig 23: Studying on the method for dsRNA extraction from plant viruses by Dr. Tomohide Natsuaki – Vice President of Utsunomiya University

4.2. Study visit to Yokohama Plant Protection Station

- Having an overview on the plant quarantine system in Japan.
- Understanding the function and operation of each division under plant quarantine system of Japan.
- Visiting to the plant quarantine facilities of Research Center of Yokohama Plant Protection Station.
- Discussing and learning on the experiences in plant quarantine field from plant quarantine officers.

Fig 24. Attending the lecture on plant quarantine system in Japan

Fig 25. Introduction of facilities and on-going activities at the Research Center

Fig 26. Visiting to the exhibition room of Yokohama Plant Quarantine Station

Fig 27. Group photo in front of Yokohama Plant Quarantine Station

4.3. *Attending the 2015 ISSAAS (International Society for Southeast Asian Agricultural Sciences) International Congress held at Tokyo University of Agriculture (Setagaya Campus)*

- Having an opportunity to gain knowledge through keynote lectures, plenary lectures, scientific presentations and parties.
- Making friend with researchers from various countries and build up network with them for possible future collaboration.

Fig 28. Memorial pictures with researchers met in ISSAAS 2015

Fig 29. Attending several activities during ISSAAS conference 2015

4.4. *Other activities*

4.4.1. *Participating in the celebration of The 124th Anniversary of Tokyo NODAI*

- Having wonderful experiences during the event, including Japanese tea ceremony, Japanese agricultural product exhibition, Japanese traditional sports, etc.
- Enjoying special foods prepared by foreign students studied in Tokyo NODAI.

Fig 30. Attending cultural activities during the *124th anniversary of Tokyo NODAI*

4.4.2. *Visiting to NODAI Food and Agriculture Museum*

- Understanding the foundation, history and development of Tokyo University of Agriculture (Tokyo NODAI).
- Interesting information on specimens of chicken variety collected over the world.
- Obtaining knowledge on Japanese culture; agriculture and method for making Sake.

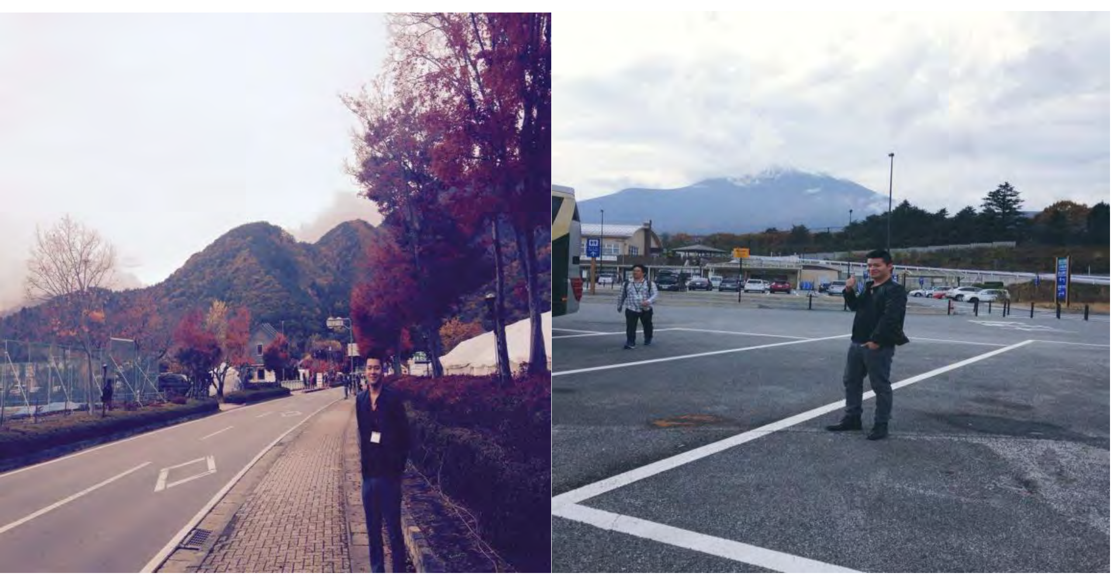
Fig 31. Photos in *NODAI Food and Agriculture Museum*

4.4.3. *Sightseeing to some famous places*

Meiji temple **NHK studio park**

Asakusa temple **Ueno Zoo**

Nikko – the world heritage



Fuji Mountain – the symbol of Japan

4.5. *What have impressed me during the two months program in Tokyo NODAI*

- The environment in Japan is always clean and neat.
- The great metro systems in Tokyoare convenient and eco-friendly.
- The Japanese are very polite, hospitable and friendly.
- Japanese students in HOGOKEN Laboratory in particular are well-knowledge in their major, laborious, helpful and friendly.

5. Summary and Recommendation

5.1. Summary

The two-month attachment program "***Training Workshop on Advanced Diagnostics on Plant Viruses"*** has been implemented in Tokyo University of Agriculture (TUA), Japan from October 27th to December 24th 2015 with three participants from Malaysia, Indonesia and Vietnam respectively. The program was under supervision by Prof. Keiko Natsuaki and Dr. Marita S. Pinili. Practical trainings have been carried out at the Laboratory of Tropical Plant Protection (HOGOKEN Lab.), Department of International Agricultural Development, TUA. During the program, I was able to learn on the tecniques and gained knowledge especially on diagnostic of plant viruses. In addition, the two days visit and hands-on work on the dsRNA extraction method at Utsunomiya University was a good exposure to the advanced technology in diagnostic of viruses. This laboratory activity was under supervision of Dr. Tomohide Natsuaki. Moreover, we were able to attend three expertise lectures on "Attenuated plant viruses" by Prof. Keiko Natsuaki; "Plant parasitic nematodes" by Dr. Marita S. Pinili and "Phylogenetic tree" by Dr. Noriko Furuya. Besides experiments at the laboratory, we were given opportunity to participate at the 2015 International Congress of International Society for Southeast Asian Agricultural Sciences (ISSAAS). In addition, the study visit to Yokohama Plant Protection Station was a good opportunity for the ASEAN participants to understand the plant quarantine system in Japan. Before the end of attachment program, a final meeting was held for the ASEAN participants to perform their final reports on the program.

In summary, I was able to improve our diagnostic skills in plant viruses and quarantine test procedures starting from symptoms observation until the identification of the plant viruses for agriculture products trade and applying the correct management techniques in order to control the spread of plant virus infections within the country.

5.2. Recommendations

The attachment program is great opportunity for myself and the other ASEAN participants to learn the advanced techniques on diagnostic of plant virus diseases from Japan – one of the top world countries in agricultural science. This program is excellent and I do not have any comments. Besides knowledge gained, I was able to see the interesting Japanese cultures and met with excellent people throughout the program. . I will try my best to apply the knowledge and experiences gained from the program in my work and share with my co-workers. I hope there are more projects collaboration between the ASEAN Plant Health Cooperation Network (APHCN) – ASEANET and Japan government for ASEAN participants in the future.

6. Acknowledgement

I would like to express my deepest appreciation to Japan-ASEAN Integrated Fund (JAIF) and the ASEAN Plant Health Cooperation Network (APHCN) of ASEANET to organize the training program for ASEAN countries' participants.

Special thanks to Dr. Lum Keng Yeang and Dr. Soetikno Sastroutomo who given me opportunity to participate in this project and helping me on the preparation for the attachment program in Japan.

In addition, I would like to express my sincere gratitude to Professor Keiko Natsuaki of Tokyo University of Agriculture (Tokyo NODAI) for her guidance, encouragement and experience sharing throughout the attachment program. Thank you for taking care our needs and accommodate us during our stay in Japan.

My sincere thanks to Dr. Tomohide Natsuaki, Dr. Marita S. Pinili and Dr. Noriko Furuya for the great lectures and practical instructions.

To all my friends from HOGOKEN Laboratory, thank you for your help and support during the experiments despite their busy schedule in studying. Last but

not least, my friends from Malaysia and Indonesia, thank you for making the training program exciting and full of fun.

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Annex 6: Detection of Potyvirus on taro

Annex 7: Detection of Banana bunchy top virus (BBTV) from Infected Banana

Annex 8: Detection of Banana bunchy top virus (BBTV) on abacca

Annex 9: Plant parasitic nematods as vectors

Annex 10: Methods of storage of virus-infected samples by PTA Plant Card

Annex 11: Detection of Banana bunchy top virus (BBTV) and Banana bract mosaic virus (BBrMV) from FTA Plant Card

Annex 12: Detection of Banana bunchy top virus (BBTV) from Viruliferous Aphids (Direct) and FTA Plant Card

Annex 13: Detection of BBTV from aphid-impregnated FTA plant card

Annex 14: Method for developing phylogenetic tree using MEGA software

Annex 15: Method for purification, ligation, transformation, cloning and sequencing of DNA of plant viruses

Annex 16: Method for extraction of dsRNA from double-stranded RNA plant viruses

Annex 17: Method for extraction of RNA of plant viruses using phenol-chloroform solution

Annex 18: Method for purification of dsRNA of plant viruses from RNA-dissolving solution

Annex 19: Detection of unknown potyvirus on passionfruit plant

Annex 20: Report on visit study to Yokohama Plant Protection Station

Annex 21: Method for SDS PAGE (Sodium Dodecyl Sulfate PolyAcrylamide) electrophoresis

Report 1. Detection of unknown virus on bamboo plant by Indirect-ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Oct. 28th, 2015

2. Material

Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

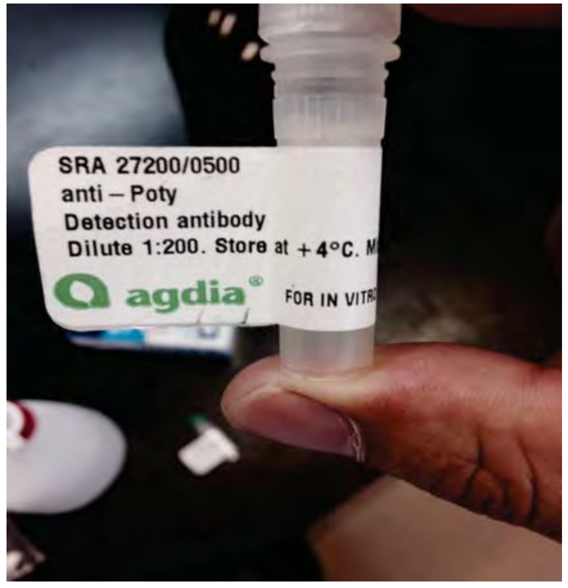


Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Bamboo (*Pleioblastus chino*) collected in Main gate of Tokyo NODAI on Oct. 28th

Fig. 2: sample 1 with symptoms: mosaic & local chlorotic spots

Fig. 3: sample 2 with symptoms: mosaic, long yellow stripe, necrosis on leaves

4. Procedure

– *Step 1: Extraction of sample*

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

– ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate
- Tap the ELISA plate to remove excess washing buffer
- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.
- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.
- Mix thoroughly.
- Add 100 µl this solution to each well.
- Incubate at room temperature under dark condition.
- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	0.064	0.068	0.073	0.079	
Positive control	0.161	0.261	0.370	0.468	
Buffer	0.065	0.066	0.067	0.070	
Sample 1	1.711	3.206	(very high)	(very high)	+
Sample 1	0.125	0.182	0.250	0.313	+

*** Average value of two wells**

6. Discussion

- Two bamboo samples collected in Tokyo University of Agriculture get positive with *Potyvirus*.
- Sample 1 has the highest absorbance value indicated that the concentration of virus in this sample is very high.

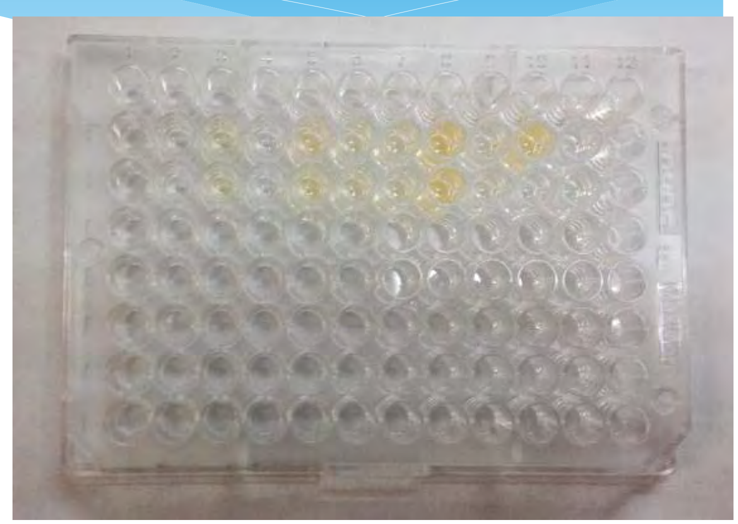
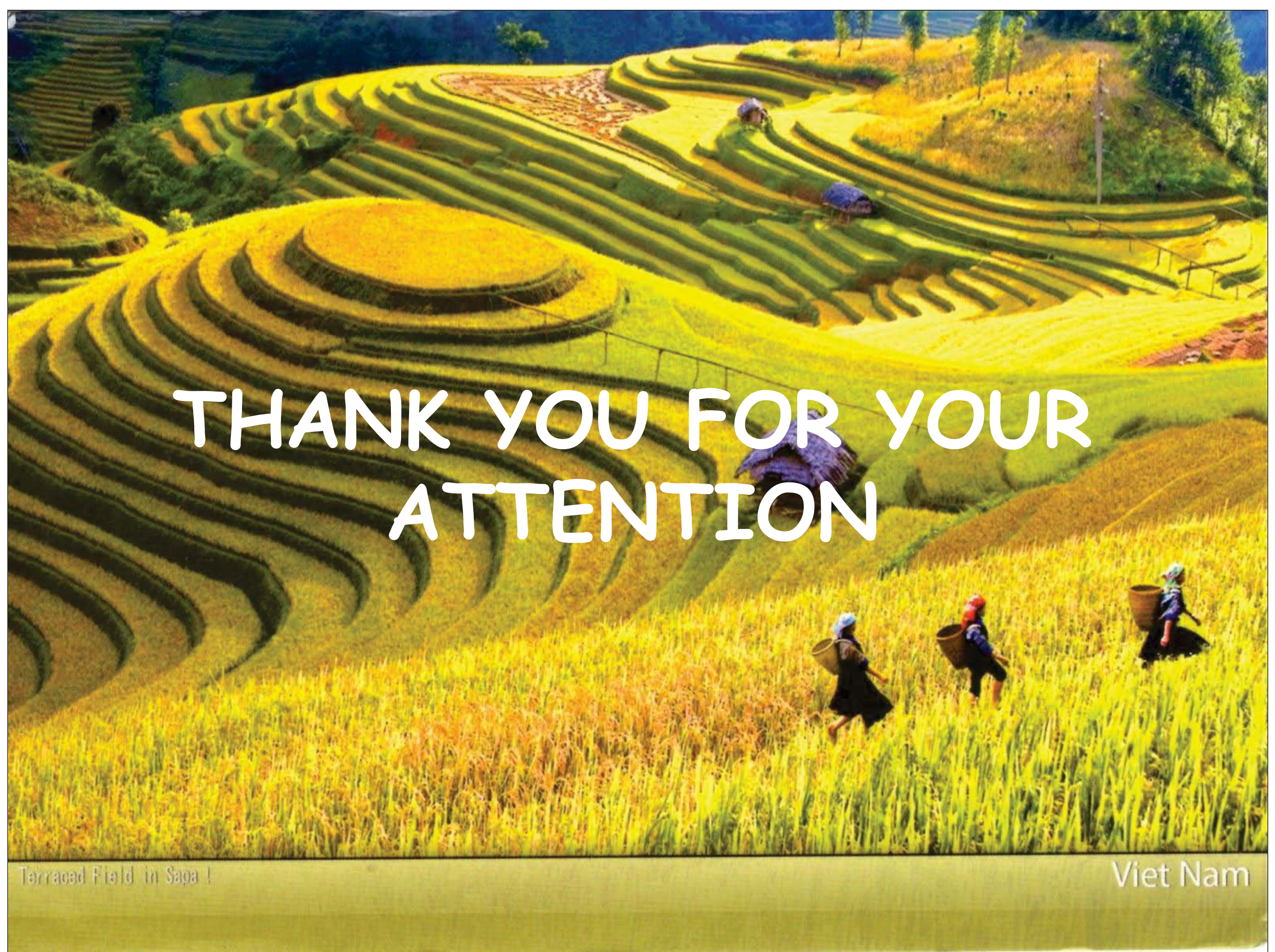


Fig. 4: result of Indirect ELISA reaction for detection of virus on bamboo. Two bamboo samples also get positive with *potyvirus*.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Report 2. Method for inoculation of unidentified passionfruit virus to passionfruit seedlings

1. PLACE AND TIME

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Oct. 29th, 2015

2. MATERIAL

- Mortars and pestles kept in freezing container of the fridge.
- Phosphate buffer 0.1 M (pH = 7.0)
- Carborundum powder.

3. SAMPLES

- Leaf of passion fruit plant suspected to be infected by a *Potyvirus*
- Healthy passion fruit seedling

Fig. 1: *potyvirus* infected leaf of passionfruit with symptom: mosaic, leaf deformation, leaf crinkle, chlorotic spots and yellow vein.

4. PROCEDURE

- Step 1:

Grind well *potyvirus* infected leaf in 0.1 M Phosphate buffer (10v/w) using mortar and pestle.

4. PROCEDURE (CONTINUED)

- Step 2:

Add carborundum powder into sap juice.

4. PROCEDURE (CONTINUED)

- Step 3:

Rub sap with carborundum powder on passionfruit seedling tenderly.

4. PROCEDURE (CONTINUED)

- Step 4:

After 3~6 min., wash away extra plant sap and carborundum from inoculated leaves by water very gently.

4. PROCEDURE (CONTINUED)

- Step 5:

Label and transfer the inoculated seedling to anti-insect chamber and observe symptom after latent period.

5. THE INFORMATION ABOUT VIRUS DISEASES ON PASSION FRUIT (*PASSIFLORA* SPP.) IN THE WORLD

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
1	Bean yellow mosaic virus	Potyvirus	Croatia	No	Groundnut, pea, chickpea, soybean, lupin, common bean…	- Aphids (more than 20 species): *Myzus persicae*, *Aphis fabae*…
2	Citrus leprosis virus	Cilevirus	Brazil	No	Citrus spp.	- Mites: *Brevipalpus phoenicis*
3	Cowpea aphid-borne mosaic virus	Potyvirus	Brazil	No	Cowpea, pea, soybean, passion fruit, groundnut...	- Aphids: *Myzus persicae* - Mechanical inoculation - Seeds
4	Cucumber mosaic virus	Cucumovirus	Australia	Yes	Chili, chickpea, cucumber, carrot, soybean, tobacco, passion fruit, tomato, potato…	- Aphids (more than 60 species): *Myzus persicae*, *Aphis fabae*, *Aphis craccivora*… -Mechanical inoculation - Seeds (19 plant species)

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
5	East Asian Passiflora Virus	Potyvirus	Japan	No	Passion fruit	- Aphids: Aphis gossypii, Hyperomyzus lactucae, Myzus persicae
6	Giant granadilla malformation virus	Begomovirus	Colombia	No	Passion fruit	-Whitefly: *Bemisia tabaci*
7	Jatropha mosaic virus	Begomovirus	Puerto Rico	No	Passion fruit	-Whitefly: *Bemisia tabaci*
8	Maracuja mosaic virus	Tobramovirus	India	No	Passion fruit	-Mechanical inoculation - Contact between plants
9	Passiflora latent virus	Carlavirus	Germany	No	Passion fruit	-Mechanical inoculation
10	Passiflora ringspot virus	Potyvirus	Ivory Coast	No	Passion fruit	- Aphids: *Aphis gossypii*, *A spiraecola* -Mechanical inoculation
11	Passiflora virus Y	Potyvirus	Australia	No	Passion fruit	- Aphids: *Aphis gossypii* -Mechanical inoculation
12	Passion flower little leaf mosaic virus	Begomovirus	Brazil	No	Passion fruit	-Whitefly: *Bemisia tabaci* -Mechanical inoculation
13	Passion fruit crinkle virus	Potyvirus	Taiwan	No	Passion fruit, soybean	- Aphids: *Myzus persicae* - Mechanical inoculation

Table 1. List of passion fruit viruses reported in the world

No	Name of virus	Genus of virus	Country of origin	Presence in Vietnam	Main host plants	Manners of transmission
14	Passion fruit green spot virus	Cilevirus	Brazil	No	Passion fruit	-Mites: Brevipalpus phoenicis
15	Passion fruit vein clearing virus	unassigned genus	Brazil	No	Passion fruit	- Unknown
16	Passion fruit yellow mosaic virus	Tymovirus	Brazil	No	Passion fruit	-Beetle: Diabrotica speciosa -Mechanical inoculation
17	Passionfruit mottle virus	Potyvirus	Taiwan	No	Passion fruit	- Aphids: Myzus persicae - Mechanical inoculation
18	Passionfruit woodiness virus	Potyvirus	Australia	No	Passion fruit	- Aphids: Aphis gossypii, Myzus persicae - Mechanical inoculation
19	Purple granadilla mosaic virus	unclassified	Brazil	No	Passion fruit	-Beetle: Diabrotica speciosa -Mechanical inoculation
20	Soybean mosaic virus	Potyvirus	Colombia	No	Soybean, passion fruit	- Aphids (more than 16 species): Myzus persicae, Aphis fabae… -Mechanical inoculation - Seeds (30% or higher) - Pollen

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Annex 3

Report 3. How to make the PTA solution and detect viruses by electron microscope

1. Place and time

- Place: HOGOKEN Lab.
- Time: Nov. 2nd, 2015

2.1. How to make the PTA (Phosphatungstic acid) solution

Materials

- Phosphatungstic acid
- Purified water
- Potassium hydroxide (KOH)

Procedure

- Weight 2 g Phosphatungstic acid
- Dissolve 2 g Phosphatungstic acid in 100 ml purified water
- Adjust pH = 7.0 by Potassium hydroxide (KOH)
- Store at 2-8°C

2.2. How to bind the plant sap on to a carbon-coated collodion membrane covered copper grid (for EMS observation)

Materials

- 2% PTA solution
- Blades
- Virus-infected leaf
- Glass slide
- Carbon-coated collodion membrane covered copper grid
- Laboratory forceps
- Laboratory pipette
- Paper tissue

Produce

Step 1:

- Use blade to take a small piece of leaf (sample) from the virus-infected leaf.

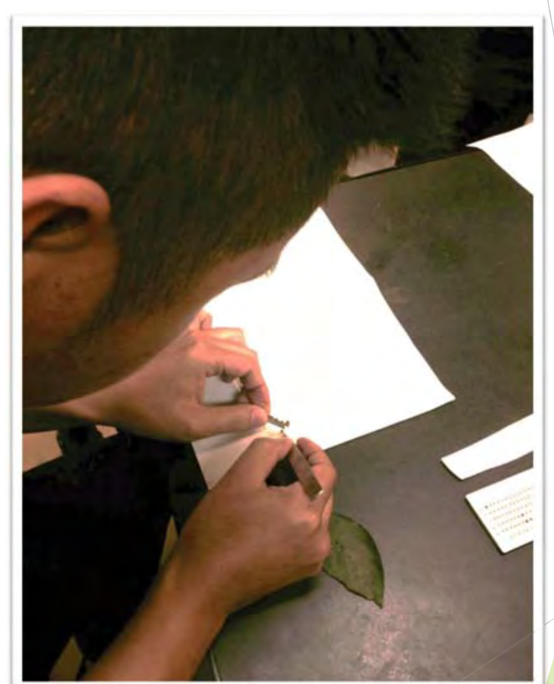
*Note: prefer taking the vein of virus-infected leaf where the concentration of virus is highest

Step 2:

- Use the laboratory forceps to transfer sample to glass slide tenderly

Step 3:

- Use laboratory pipette to drop a small amount of PTA solution on to glass slide.
- Use the blade to crush the sample with PTA solution to make the plant sap (*this activity should be done within 10-15 seconds*).



Step 4:

- Pick up the Carbon-coated collodion membrane with laboratory forceps and gently dip it in plant sap.
- Exclude the excess of plant sap by paper tissue

Step 5:

- Transfer carefully the membrane dipped plant sap to Grid storage box and prepare for EMS observation

Annex 4

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON INTRODUCTION ABOUT PLANT VACCINE OF *PEPPER MILD MOTTLE VIRUS*

1. Place and time

Place: HOGOKEN meeting room

Time: Nov. 2nd, 2015

2. Materials

The video on introduction about plant vaccine of *Pepper mild mottle virus* (PMMoV)

3. Contents

- Introduction about the production of bell pepper (*Capsicum annuum*) in Japan.

- Introduction about *Pepper mild mottle virus* (PMMoV) and its damage on bell pepper in Japan.

- How to produce the PMMoV-vaccinated seedlings

- Yield comparison between healthy, PMMoV-infested and PMMoV-vaccinated plants after harvesting.

- Vitamim C comparison between fruits collected from healthy and PMMoV-vaccinated plants

4. Discussion about the difficulty in applying vaccinated plants in our countries

***In Vietnam:** In my opinion, the most important difficulty in applying plant vaccines in Vietnam is economic aspect. Vietnam now is the developing country, so the famers are still poor. Their optimum management method for plant pests and diseases in general, and virus diseases in particular is chemical control. Therefore, it's very hard to persuade the famers to use the vaccinated plants instead of chemical. In addition, the habit of cultivation in Vietnam is different from Japan. Almost Vietnamese famers sow the plants on the fields (not in the green house), so not only

the virus diseases can attack the plants but also many pathogenic agents (fungi, bacteria, nematodes…) as well as other pests. Consequently, it's difficult to use the vaccinated plants in Vietnam. However, I hope in the near future, when the Vietnamese famers have more money and more knowledge, they can use the vaccinated plants in their cultivation as one of advanced management controls.

*** In other countries (Indonesia, Malaysia and Uganda):** The participants from these countries also said that they have had the same difficulty with Vietnam. However, Mr. Patrick believed that it would be possible if applying the vaccinated plants in vegetable cultivation in Uganda.

Report 5. Detection of Potyvirus on Passionfruit plant by Indirect ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 4th, 2015

2. Material

- Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

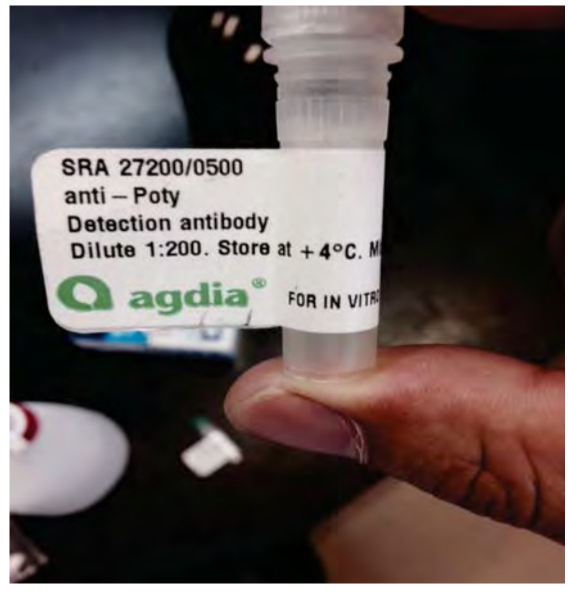


Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Potyvirus-inoculated Passionfruit seedling (date of inoculation: Oct. 29th, 2015)

Fig. 2: *Potyvirus*-inoculated Passionfruit seedling without typical symptoms.

4. Procedure

– *Step 1: Extraction of sample*

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

– ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate
- Tap the ELISA plate to remove excess washing buffer
- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.
- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

– ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.
- Mix thoroughly.
- Add 100 µl this solution to each well.
- Incubate at room temperature under dark condition.
- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

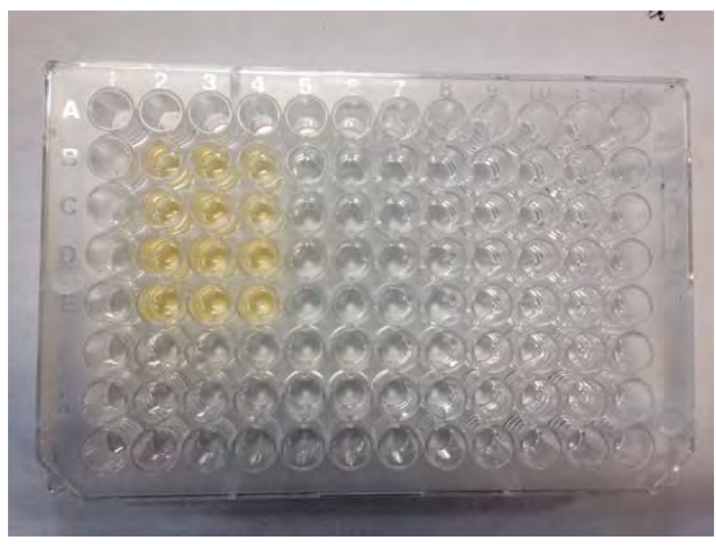
Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	0.071	0.073	0.076	0.079	
Positive control	0.073	0.083	0.096	0.116	
Buffer	0.071	0.072	0.073	0.080	
Sample 1	0.062	0.099	0.125	0.165	+

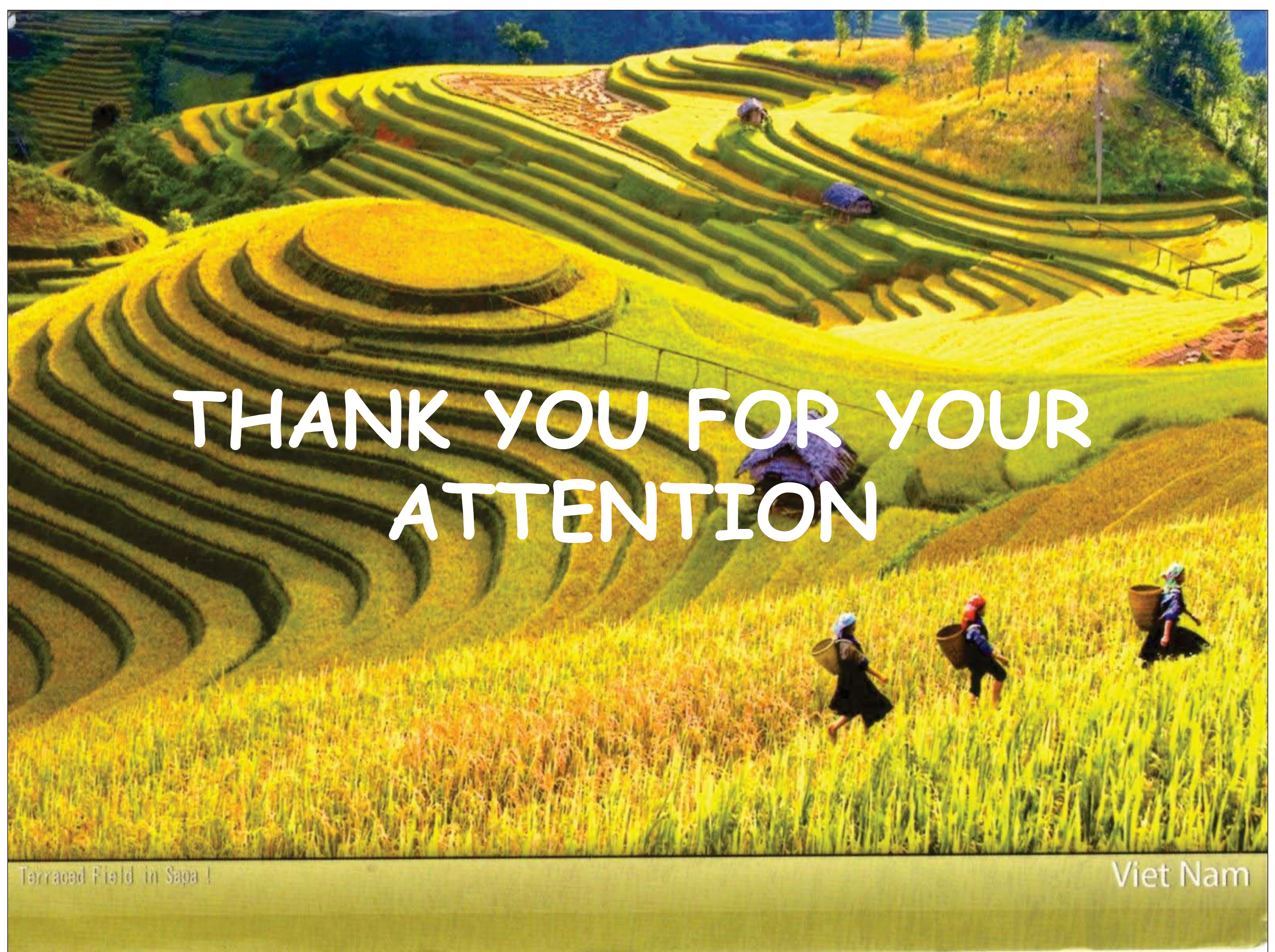
** Average value of two wells*

6. Discussion

- All the ELISA wells (includes Negative control and Buffer) turned yellow color

- This result may be explained that: the unspecific binding was occurred in Step 2 or the contamination was happened during conduct the In-ELISA process.

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Report 6. Detection of *Potyvirus* on Taro plant by Indirect ELISA

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 5th, 2015

2. Material

Indirect ELISA Kit (SRA 27200/0500) for detection of *Potyvirus* provided by Agdia company.

Fig. 1: Indirect ELISA kit (SRA 27200/0500) for detection of *Potyvirus* (Agdia, USA).

3. Samples

Fig. 2: Taro leaf with symptoms: yellow mottle and necrosis on the margin of leaf (sample 1)

Fig. 3: Taro leaf with symptoms: yellow mottle and necrosis on the margin of leaf (sample 2)

Fig. 4: yellow mottle on the margin of leaf, mosaic and leaf malformation (sample 3)

4. Procedure

- ***Step 1: Extraction of sample***

- Weigh 0.1 g sample (bamboo leaf) and transfer to the mortar. Add the nitrogen with liquid form.

- Add 1.0 ml sample extraction buffer (1X) into the mortar and grind.

- Centrifuge the plant sap at 15.000 rpm within 5 minutes.

- Load 200 µl plant sap per well of the ELISA plate.

- Incubate at Room temperature for 1 hour under dark condition.

4. Procedure (continued)

- ***Step 2: To bind detection antibody***

- After incubation, remove the plant sap from the wells by ELISA-washing machine.

- Gently tap the ELISA plate on paper tissue to make ELISA wells dry totally but not too long time.

- Add 200 µl Poty-specific antibody with a dilution of 1:200 in ECI buffer (1X) to each well.

- Incubate at room temperature for 2 hours under dark condition.

4. Procedure (continued)

- ***Step 3: To bind Enzyme-linked antibody (secondary antibody)***

- Wash the ELISA plate

- Tap the ELISA plate to remove excess washing buffer

- Add 200 µl Enzyme-linked antibody with a dilution of 1:200 in 1X ECI buffer to each well.

- Incubate at room temperature for 1 hour under dark condition.

4. Procedure (continued)

- ***Step 4: To add PNP tablet and result reading***

- Dilute 1 PNP tablet (0.5 mg) with 5 ml PNP buffer.

- Mix thoroughly.

- Add 100 µl this solution to each well.

- Incubate at room temperature under dark condition.

- Read the absorbance of ELISA plate using ELISA Reader (Microplate Reader/ Bio-RAD) after 15, 30, 45 and 60 minutes of incubation.

5. Result

Sample	Absorbance value of ELISA plate*				Conclusion
	15 minutes	30 minutes	45 minutes	60 minutes	
Negative control	-	-	-	-	
Positive control	-	-	-	-	
Buffer	-	-	-	-	
Sample 1	-	-	-	-	
Sample 2	-	-	-	-	
Sample 3	-	-	-	-	

6. Discussion

- All the ELISA wells (includes Negative control and Buffer) turned yellow color.
- This result may be explained that: the unspecific binding was occurred in Step 2 or the contamination was happened during conduct the In-ELISA process.

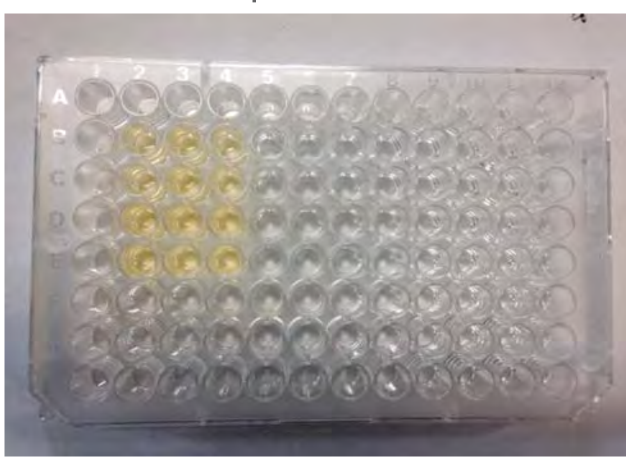

Fig. 5: result of Indirect ELISA reaction for detection of virus on taro plant. All the ELISA wells (includes Negative control and Buffer) turned yellow color.

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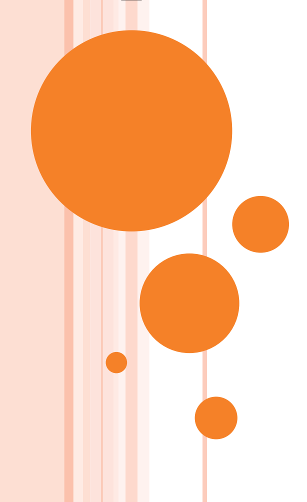
Report 7: Detection of Banana Bunchy Top Virus (BBTV) on Banana by PCR



1. Place and Time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 11th - 13th, 2015

2. MATERIALS

- DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).
- Specific PCR primers for BBTV (D11 – forward/ D12 – reverse) (Karan et al., 1994).

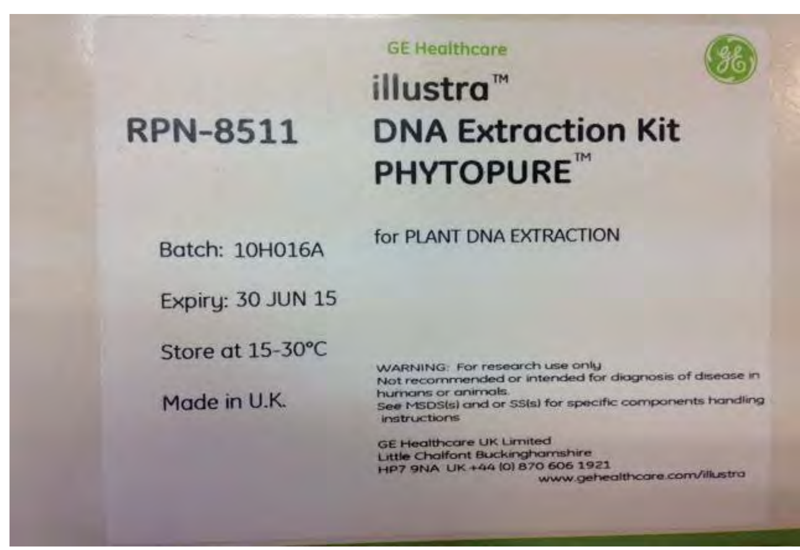


Fig 1: DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).

3. SAMPLES

Fig. 2. The BBTV inoculated- plant without symptoms

Fig. 3. Healthy plant

3. SAMPLES

Fig. 4. Banana plant used to rear banana aphid (*Pentalonia nigronervosa*)

Fig. 5. BBTV-inoculated banana plant with typical symptoms: stunt, bunchy top, yellow (chlorotic/ necrotic) leaf margins

4. PROTOCOLS FOR DNA EXTRACTION

- Step 1:

- Weigh 0.1 g sample and pulverize in liquid Nitrogen quickly using a mortar and pestle.

- Transfer the plant sap powder into new 1.5 ml tube.

4. PROTOCOLS FOR DNA EXTRACTION

- Step 2:

- Add 300 µl plant DNA extraction Reagent 1 into tube with plant sap powder, turn the tube upside down or gently shake.

4. PROTOCOLS FOR DNA EXTRACTION

- Step 3:

- Add 100 µl plant DNA extraction Reagent 2 into tube with plant sap powder and Reagent 1, turn the tube upside down or gently shake.

4. Protocols for DNA extraction

- Step 4:

- Vortex the tube for few second and heat for 10 minutes at 65°C (Dry Thermo Unit).

- Put the tube in cold box for 20 minutes

4. Protocols for DNA extraction

- Step 5:

- Add 250 µl Chloroform into tube and gently shake.

- Then, add 50 µl Resin into tube (note: pre-vortex the resin before using to avoid sedimentation)

4. PROTOCOLS FOR DNA EXTRACTION

- Step 6:

- Shake tube for 10 minutes in sharking machine (Bio Shaker BR-15LF/ TAITEC) at 25.5°C (note: check carefully the tube cap to ensure that it has been closed tightly).

4. PROTOCOLS FOR DNA EXTRACTION

- Step 7:

- Centrifuge at 2500 rpm for 10 minutes at room temperature (KUBOTA 3300). Repeat centrifugation if plant debris are not completely settled.

- Collect the supernatant (about 250 μl) and transfer into new 1.5 ml tube (avoid the plant tissue debris).

4. Protocols for DNA extraction

- Step 8:

- Add 250 µl 2-propanol and shake gently (note: the volume of 2-propanol must be equal with collected supernatant in step 11).

- Centrifuge at 15,000 rpm for 5 minutes at room temperature. The DNA pellet will be precipitated at the bottom of tube.

4. Protocols for DNA extraction

- Step 9:

- Pipette out the liquid carefully and ddd 100 µl 70% ethanol and gently wash the tube.

- Centrifuge at 15,000 rpm for 2 minutes at room temperature, then discard carefully as much ethanol as possible

4. Protocols for DNA extraction

- Step 10:

- Air-dry for 2-3 minutes at room temperature

- Add 100 µl 1X TE buffer and break the DNA pellet by touching with a pipette tip.

5. Protocols for PCR assay

- Step 1: Prepare the cocktail mixture; calculate the required amount as follow:

	For detection	For sequencing
q.s.	17.4 µl	34.8 µl
10x Ex Taq Buffer	2.5 µl	5.0 µl
dNTP mixture	2.0 µl	4.0 µl
Forward primer (25 pmol)	0.25 µl	0.5 µl
Reverse primer (25 pmol)	0.25 µl	0.5 µl
TaKaRa Ex Taq (5 units/µl)	0.1 µl	0.2 µl
Template DNA	2.5 µl	5.0 µl
Total	25 µl	50 µl

5. PROTOCOLS FOR PCR ASSAY

- Step 2:

- Transfer 22.5 µl the cocktail mixture into PCR tube, then add 2.5 µl of extracted DNA.

- Flash PCR tube for few seconds (must not have the bubbles).

- Conduct PCR assay by PCR machine (DNA Engine/ BioRAD) with above given PCR conditions.

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5. PROTOCOLS FOR PCR ASSAY

- Step 2:

- Transfer 22.5 µl the cocktail mixture into PCR tube, then add 2.5 µl of extracted DNA.

- Flash PCR tube for few seconds (must not have the bubbles).

5. Protocols for PCR assay

- Step 3: Conduct PCR assay by PCR machine (DNA Engine/ BioRAD) with above given PCR conditions.

6. Protocols for *Gel electrophoresis*

- Prepare 2% agarose gel in 1X TAE buffer.
- Completely dissolve the agarose in the buffer using a microwave.
- Let the solution to slightly cool down (~ 5 minutes).
- Pour the solution slowly into the casting tray with the comb in place. Avoid forming any bubbles.
- Let the agarose get to solidify (~30 – 45 minutes) then carefully remove the comb.
- Place the solidified agarose gel into the electrophoresis unit. Fill in with 1X TAE buffer until the gel is fully submerged.

6. Protocols for Gel electrophoresis (continued)

- Put 2 µl blue juice in a piece of parafilm.
- Mix 13 µl PCR product (for 6 band-comb) into blue juice by carefully pipetting the solution in and out of the tip. Avoid forming any bubbles. (For 8 bands (small comb) use 8 µl PCR product with 2 µl blue juice).
- Load the sample mixture into gel starting with 100 bp ladder/marker (for BBTV) at 15 µl; followed by the negative control.
- Run samples for 25 – 30 minutes using electrophoresis machine (Mupid-2plus/ Advance).
- After running, stain the gel by submerging it into Ethidium bromide solution for 5 minutes.
- De-stain the gel in distilled water for 1 – 2 minutes.
- View DNA band under UV illumination and take photo using EDAS 290 (Kodak, Japan).

7. Result

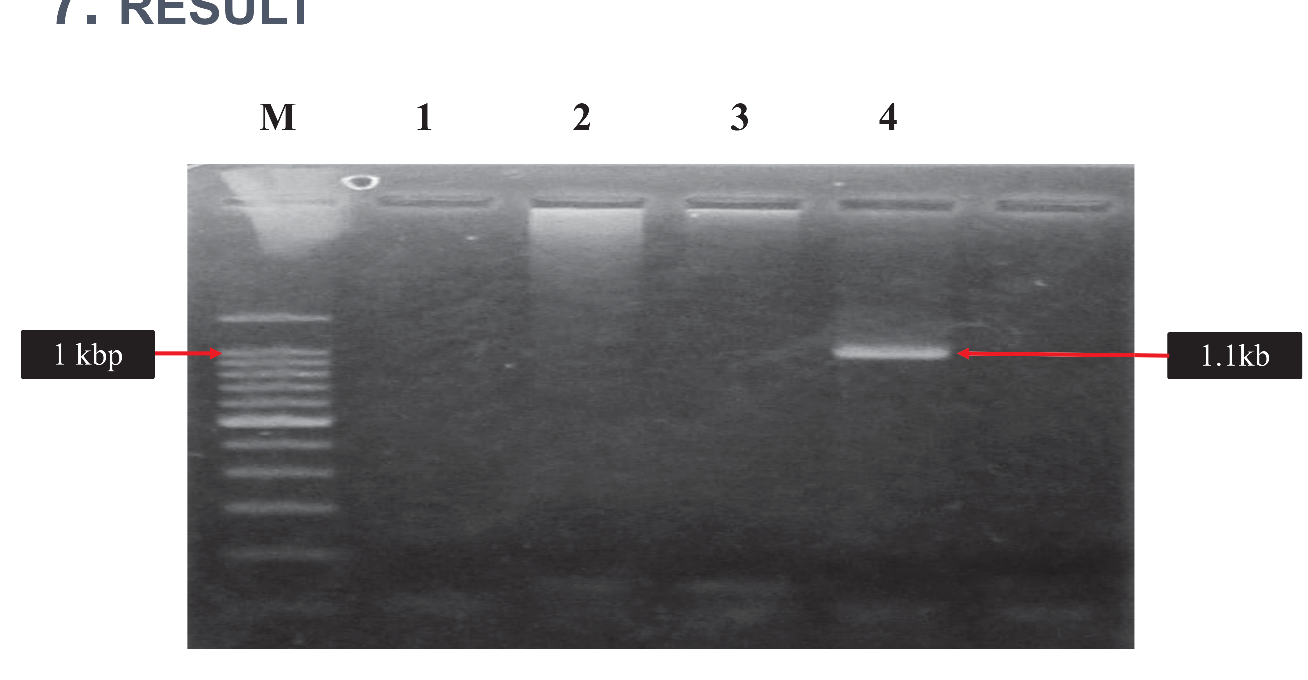

Fig 4. PCR assay of *Banana bunchy top virus* (BBTV) collected from HOGOKEN Lab. with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 4 (lane 4). No band was amplified from Sample 1-3 (lanes 1-3). The 100bp DNA ladder (Promega, USA) was included as marker.

8. DISCUSSION

- The BBTV-inoculated banana plant (sample 4) shows symptoms: stunt, bunchy top, yellow (chlorotic/ necrotic) leaf margins (Fig. 5) resulted positive to BBTV presence. Sample 2 (BBTV-inoculated banana plant without symptoms – Fig. 2) as well as other samples gave negative to BBTV.

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Report 8. Detection of Banana Bunchy Top Virus (BBTV) on Abaca (*Musa textilis*) by PCR

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
  - Time: Nov. 12th - 16th, 2015

2. Material

- DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).
- Specific PCR primers for BBTV (D11 – forward/ D12 – reverse) (Karan et al., 1994).

Fig 1: DNA extraction Kit PHYTOPURE (RNP-8511) (GE healthcare, USA).

3. Samples

Fig. 2: the samples of Abaca (*Musa textilis*) collected in the Philippines in 2011 and was kept at (-) 30°C in HOGOKEN Lab. Sample 1: Positive control for BBTV; Sample 2: was named "Abaca forestry"

4. Procedure for DNA extraction, PCR assay and gel electrophoresis

– **Procedure for DNA extraction, PCR assay and gel electrophoresis was described in previous report (see report no. 7)**

5. Result

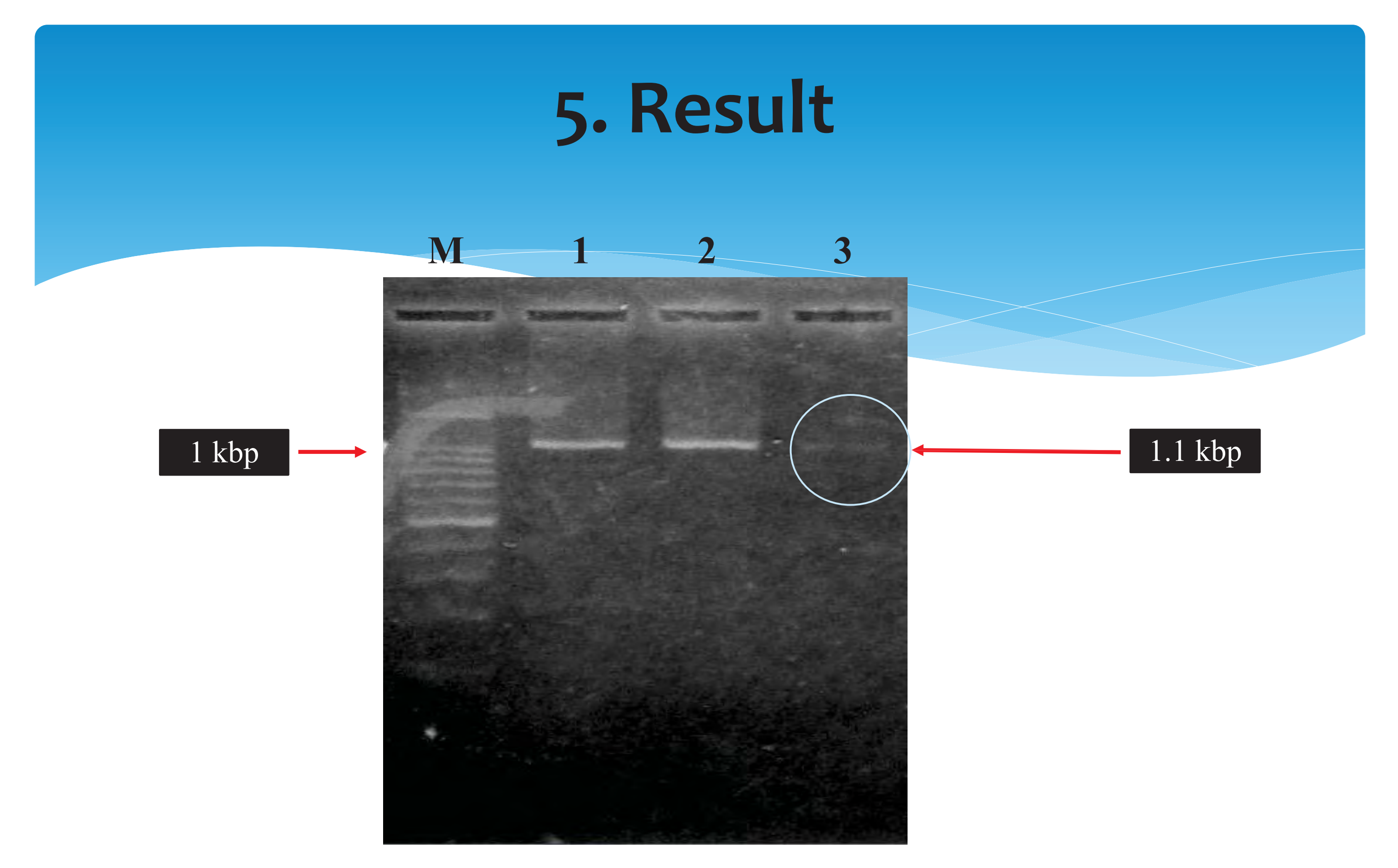

Fig 3. PCR assay of *Banana bunchy top virus* (BBTV) on Abaca (*Musa textilis*) collected from the Philippines with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrows) was amplified from Positive controls (lanes 1-2). The PCR band in was amplified from Sample 2 (lane 3) was unclear (in circle). The 100bp DNA ladder (Promega, USA) was included as marker.

6. Discussion

– The sample 2 named "Abaca forestry" (Fig. 2) resulted positive to BBTV presence. However, the PCR band was unclear. This result may be caused due to reduction of concentration of virus during storage period.

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON LECTURE ABOUT PLANT PARASITIC NEMATODES AS THE VECTOR FOR PLANT VIRUSES

1. Lecturer

Dr. Marita S. Pinili, from IPB, University of the Philippines Los Banos

2. Place and time

Place: HOGOKEN meeting room, Tokyo University of Agriculture (Tokyo NODAI).

Time: Nov. 12th, 2015

3. Contents

3.1. Introduction about plant nematodes

3.1.1. General morphological characteristics

Nematodes are worm shape (vermiform), unsegmented, pseudoceolomate, multicellular, triploblastic, and bilaterally simetrical. Nematodes were once classified with a very large and heterogeneous cluster of animals grouped together on the basis of their overall worm-like appearance, simple structure of internal body set, and well-defined head.

3.1.2. Biological characteristic

Life cycle of nematode parasitic plants are: egg, juvenile, and adult. Egg hatched as a juvenile. There are four juvenile stadia, separated another by molt. Juvenile is the most damaged stadia of nematode in plant. Major type nematodes feeding strategies/parasitic habit nematode are:

1. Ectoparasites in which the nematode remains outside of the plant and uses its stylet to feed from the cells of the plant roots

2. Semi-endoparasites nematodes are able to partially penetrate the plant and feed at some point in their life cycle
3. Migratory parasites nematodes can spend much of their time migrating through root tissues destructively feeding on plant cells
4. Sedentary endoparasites. The most damaging nematodes in the world have a sedentary endoparasitic. The two main nematodes in this group are the cyst nematodes (*Heterodera* and *Globodera*) and the root-knot nematodes (*Meloidogyne*)
5. Stem and bulb nematodes (*Ditylenchus* spp.) are, as their name suggests, nematodes that attack the upper and lower parts of plants
6. Seed gall nematodes (*Anguina* spp.) . These nematodes migrate as J2s in water films to the leaves of plants where they feed as ectoparasites at the tips, causing distortion of the leaves.
7. Foliar nematodes are in the genus *Aphelenchoides*. The adult nematodes migrate in water films on the stems to the leaves of their host plant and penetrate the leaves through natural openings (stomata)

3.1.3. Damages caused by plant nematodes

There are two type of nematodes which are free-living nematodes and plant parasitic nematodes. Roles nematodes in disease development there are as a pathogen, incitant, and vector of virus. Nematodes pathogen can cause disease even if the absence of other organism. Nematodes can attack healthy plants tissues that creates infection courts for other organism and causes minimal damage. Nematodes as a vector can carries other pathogen in host tissues but it is not further involved in disease development.

3.2. Plant parasitic nematodes as the important vectors of plant viruses

3.2.1. Defined genera of plant parasitic nematodes as the vector of plant viruses.

Genera of nematodes that carries plant viruses are Triplonchida and Dorylaimida. Triplonchida transmit *Tobravirus* (*Trichodorus* sp., *Paratrichodorus* sp.), while Dorylaimida can transmits genus of *Nepovirus* (*Xiphinema* sp, *Longidorus* sp., and *Paralongidorus* sp.).

Tobravirus derived from *Tobacco Rattle Virus* and have straight tubular particle. All *Tobravirus* species are ssRNA positive-strand (+ss RNA) viruses. Some other important *Tobravirus* are *Pea early-browning virus* (PEBV) and *Pepper ringspot virus* (PepRSV). While *Nepovirus* derived from *Nematode Polyhedral Virus* which have isometric particle of 28 nm diameter, and have bipartite genome with two functional RNA molecules. They are ssRNA positive-strand (+ss RNA) viruses. Some other important *Nepovirus* are *Tobacco ringspot virus* (TRSV); *Grapevine fanleaf virus* (GFLV) and *Beet ringspot virus* (BRSV).

3.2.2. Manner of transmission of plant viruses by plant parasitic nematodes.

Plant viruses are transmitted by plant parasitic nematodes following non-persistent manner. Virus cannot replicate in nematodes body, not passed transovarily through nematode eggs, and is eliminated from nematode body when juvenile's molt.

- Ingestion : the intake of virus particles during feeding
- Acquisition: act of ingesting virus particles
- Absorption: the active of process by which virus particles adhere to specific sites of retention in the feeding apparatus

- Retention : the period during which specifically absorbed attached of the site retention in the feeding apparatus
- Release: the dissociation of the virus particles from the specific site of retention in the feeding apparatus
- Transfer: The placement of virus particles in plant cell

3.4. Method for conducting the experiments on virus-transmission by plant parasitic nematodes and detection of virus in nematodes.

- Extraction nematode from rhizospere of infected plant
- Add nematode to healthy bait plant grown in sterilized soil
- Transmission confirmed by manifestation of similar symptom

Detection and identification of virus can be done by serology methods (ELISA, ISEM) or molecular method (molecular hybridization technique, PCR, and RT-PCR).

Report 10. Method for storage of virus-infected samples by FTA plant card

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 16th, 2015

2. Materials

- *FTA plant card*

Fig. 1: The FTA plant cards can be purchased from Whatman company (GE healthcare Life Science). This plant cards are easy to be used and stored in Laboratory.

3. Samples

Fig. 2: The BBTV-infected samples were collected in the Philippines in 2011 and stored at (-) 30°C in HOGOKEN Lab.

4. Produce

Step 1:

- Use scissor to cut sample into small pieces and transfer to the mortar.

Step 2:

- Add 200 µl sterilized water into mortar and grind the sample by pestle.

Step 3:

- Use the pipet to transfer the plant sap into FTA plant card (**note:** do not pipet the plant sap out of the round cycle of plant card to avoid the contamination with other samples).

Step 4:

- Let FTA plant card dry under room temperature within 1 hour.
- Put the FTA plant card with plant sap in plastic bag with silicagen and keep in cool dry place. The plant sample will be stored by FTA plant card more than 1 year.

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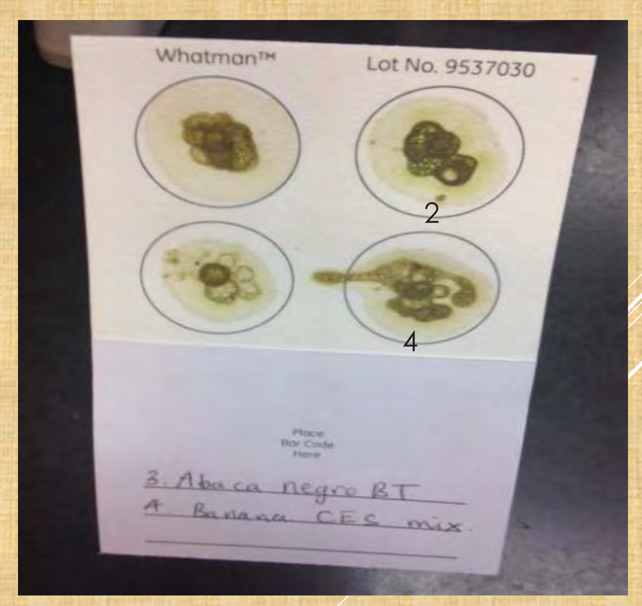
Report 11. Detection of Banana Bunchy Top Virus (BBTV) and Banana Bract Mosaic Virus (BBrMV) from FTA plant card.

1. PLACE AND TIME

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 16th - 17th, 2015

2. SAMPLES AND MATERIALS

- *2.1. Samples*
- \- FTA plant card with banana and abaca samples which are infected by BBTV and BBrMV. These samples were collected in the Philippines in 2011 by Dr. Marita Pinili (IPB, University of the Philipoines Los Banos) and stored at (-) 30°C in HOGOKEN Lab.



Sample 2. Abaca Forestry isolate

Sample 4. Banana CES mixed infection

2. SAMPLES AND MATERIALS

- *2.2. Materials*

\- FTA plant Kit (GE healthcare Life Science, UK)

(1) FTA membrane puncher; (2) Cutting mat; (3) FTA purification reagent

3. PROTOCOLS

3.1. Extraction of total plant DNA from FTA plant card

- Step 1:

- Use puncher to take 8 pieces of sample on FTA plant card and transfer into 1.5 ml eppendorf tube (<u>note:</u> should not take much plant tissue debris).

- Step 2:

- Add 100 µl processing buffer, then add 1 µl RNAse inhibitor into 1.5 ml tube.

- Then, incubate on ice with mixing every 5 minutes interval for 30 minutes.

- Step 3:

- Transfer the supernatant into new 1.5 ml tube and add 10 µl 3M Sodium acetate (pH = 5.2) and 10 µl of cold 2-propanol.

- Incubate at (-) 80°C (Panasonic, Japan) for 30 minutes.

- Step 4:

- After finishing the incubation, centrifuge the tube at 15,000 rpm (KUBOTA 3300) for 10 minutes.

- Step 5:

- Discard supernatant and wash total plant DNA pellet with 500 µl 75% Ethanol.

- Centrifuge the tube at 15,000 rpm (KUBOTA 3300) for 02 minutes.

- Step 6:

- Remove the Ethanol by pipet, then dry tube at room temperature for 2-3 minutes.

- Dissolve total plant DNA pellet in 30 µl DEPC-treated water and store at (-) 30°C for next using.

3.2. Synthesis of cDNA (for BBrMV)

- Prepare the cocktail mixture in PCR tube; calculate the required amount as follow:

5X RT buffer	4.0 µl
dNTP mixture (10 mM)	2.0 µl
Reverse primer of BBrMV	1.0 µl
RNAse inhibitor (10U/ µl)	1.0 µl
ReverTra Ace™	1.0 µl
Total RNA	11 µl
Total	**20 µl**

3.2. Synthesis of cDNA (for BBrMV)

- Conduct synthesis of cDNA by PCR machine (DNA Engine/ BioRAD) with PCR conditions below:

Temperature (°C)	Time (min.)
42	20
99	5
4	endless

3.3. PCR assay and Gel electrophoresis

- Conduct PRA assay with PCR conditions as follow:
- For BBTV

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

3.3. PCR assay and Gel electrophoresis

- For BBrMV

Temperature (°C)	Time (min.)	Cycles
94	1	
94	0.5	32
61	1	
72	1	
72	3	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. RESULT

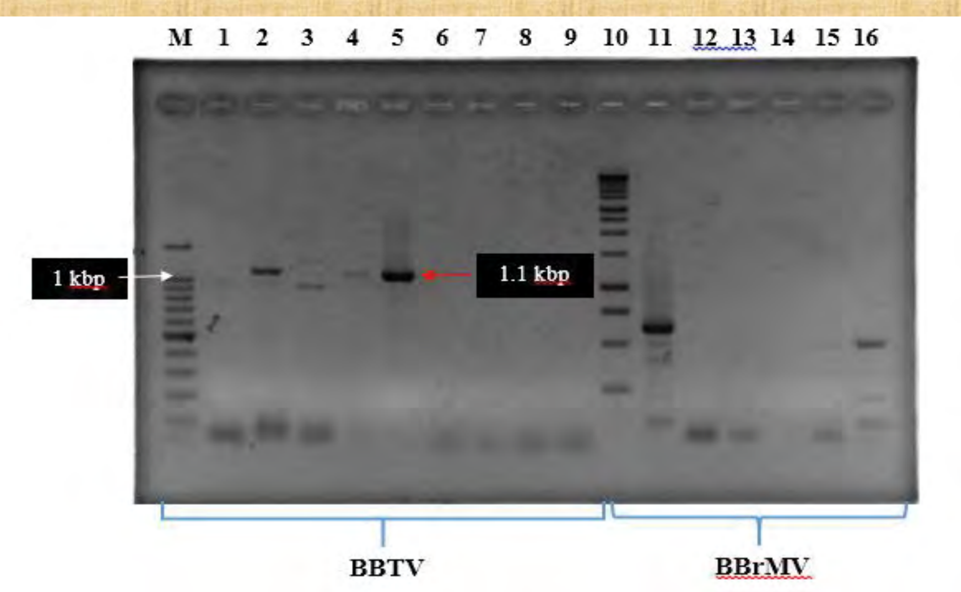


Fig 1. PCR assays on frozen samples collected in the Philippines in 2011 (Abaca Forestry isolate – lane 2; 13 and Banana CES mixed infection – lane 4; 14) with D11/D12 primer pairs for *Banana Bunchy Top Virus* (BBTV) and Bract 1/ Bract 2 for *Banana Bract Mosaic Virus* (BBrMV). For BBTV, the PCR band with approx. size of ~ 1.1kb (red arrow) was amplified from both samples (lane 2; 4). For BBrMV, no band was amplified from these samples (lane 12-13). The 100bp DNA and 1kb DNA ladder (Promega, USA) was included as marker.

5. DISCUSSION

- Both samples (Abaca Forestry isolate and Banana CES mixed infection) resulted to positive with BBTV, whereas got negative with BBrMV.
- These samples which has been kept at (-) 30°C for over 4 years still have a good concentration of virus for PCR assay. It showed that making plant samples in freezing condition at (-) 30°C or below is one of the best ways to store virus-infected samples.

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Report 12. Detection of Banana Bunchy Top Virus (BBTV) from Aphid

1. PLACE AND TIME

Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).

Time: Nov. 18th – 19th, 2015

2. SAMPLES AND MATERIALS

2.1. Samples

– The banana aphid (*Pentalonia nigronervosa*) samples were collected from banana and taro plant in HOGOKEN Lab. 1 week ago and kept in 1.5 ml tube with 70% Ethanol.

Sample 1. The aphids were collected from BBTV-infected banana plant.

Sample 2. The aphids were collected from BBTV-inoculated banana plant but not shown symptoms.

Sample 3. The aphids were collected from BBTV-inoculated taro plant but not shown symptoms.

2. SAMPLES AND MATERIALS

2.2. Materials

– DNA extraction Kit (GE healthcare Life Science, UK)

3. PROTOCOLS

3.1. Extraction of total DNA from banana aphid

1. Immobilize 15-20 aphids by 70% Ethanol.
2. Remove the Ethanol and dry the aphid by tissue/ filter paper at room temperature.
3. Transfer the aphids into cold mortar and grind in liquid nitrogen.
4. Collect the sample quickly and place in 1.5 ml tube.
5. Perform DNA extraction following the procedure of Nucleon Phytopure DNA Extraction Kit (GE healthcare Life Science, UK).

3.2. PCR assay and Gel electrophoresis

Conduct PRA assay with PCR conditions as follow:

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. RESULT

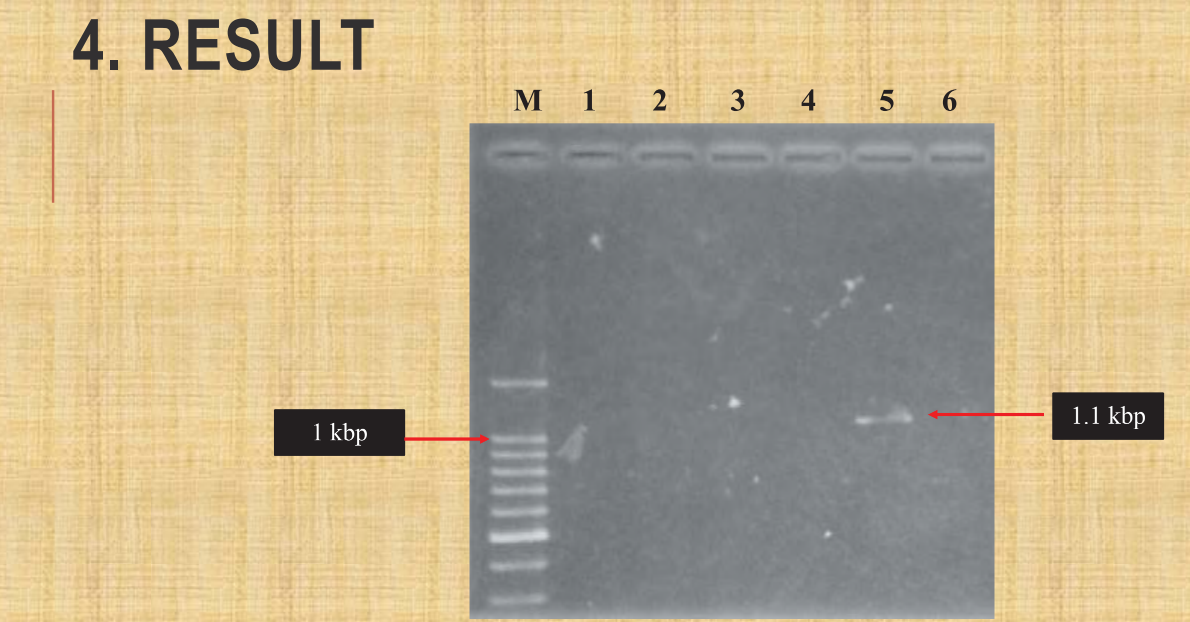

Fig 1. PCR assay of *Banana bunchy top virus* (BBTV) on banana aphid with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 1 (lane 5). No band was amplified from Sample 2 and 3 (lanes 3; 4; 6, respectively). Lane 2 was negative control. No band in lane 1 (replication test of sample 1). The 100bp DNA ladder (Promega, USA) was included as marker.

5. DISCUSSION

The aphid sample collected BBTV infected banana plant (sample 1) resulted positive to BBTV presence. Sample 2 (aphids on BBTV-inoculated banana plant) and Sample 3 (aphids on BBTV-inoculated taro plant) gave negative to BBTV. There was no band in lane 1 (replication test of sample 1). This reason might be occurred because of problem in DNA extraction step. There was no total DNA of sample 1 obtained after this step.

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Report 13. Detection of BBTV from aphid-impregnated FTA plant card.

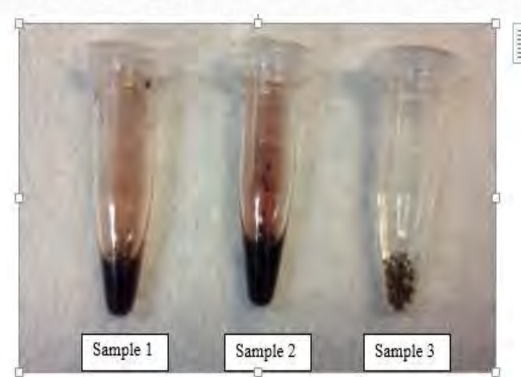
1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 18th - 19th, 2015

2. Samples and materials

- *2.1. Samples*

- The banana aphid (*Pentalonia nigronervosa*) samples were collected from banana and taro plant in HOGOKEN Lab. 1 week ago and kept in 1.5 ml tube with 70% Ethanol.



Sample 1. The aphids were collected from BBTV-infected banana plant.

Sample 2. The aphids were collected from BBTV-inoculated banana plant but not shown symptoms.

Sample 3. The aphids were collected from BBTV-inoculated taro plant but not shown symptoms.

2. Samples and materials

- *2.2. Materials*

- FTA plant card (GE healthcare Life Science, UK)

2. Samples and materials

- *2.2. Materials*

- FTA plant Kit (GE healthcare Life Science, UK)

(1) FTA membrane puncher; (2) Cutting mat;

(3) FTA purification reagent

3. Protocols

3.1. Impregnating of aphid's nucleic acid into FTA plant card

- Step 1:

- Immobilize 15-20 aphids by 70% Ethanol.

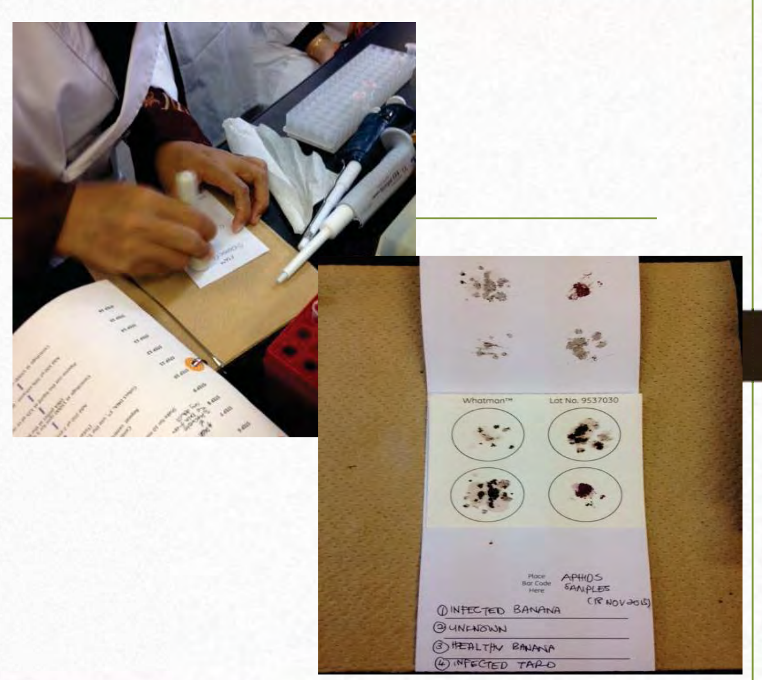
- Remove the Ethanol and dry the aphid by tissue/ filter paper at room temperature.

- Step 2:

- Place the aphid in membrane of FTA plant card.

- Step 3:

- Fold the cover of FTA plant card, then macerate the aphid by pestle till the nucleic acid impregnate into the card.



3.2. Extraction of DNA of Aphid from FTA plant card

1. Use the puncher to take 1-2 disks from the FTA plant card, then put into PCR tube.
2. Add 200 µl 90% Ethanol into the PCR tube and incubate for 5 minutes at room temperature.
3. Remove the Ethanol and add again 200 µl 90% Ethanol and incubate for 30 minutes at room temperature.
4. Add 200 µl FTA purification reagent and incubate for 5 minutes at room temperature.
5. Remove the reagent and repeat step 4 two times more.
6. Add 200 µl 1X TE buffer (pH = 8.0) and incubate for 5 minutes at room temperature.
7. Repeat step 6.
8. Remove the liquid and dry the disks for 1 – 2 hours at room temperature.
9. Use these disks for PCR assay or store at (-) 30°C for next using.

3.3. PCR assay and Gel electrophoresis

- Conduct PRA assay with PCR conditions as follow:

Temperature (°C)	Time (min.)	Cycles
94	4	
94	1	29
61	1	
72	2	
72	10	

- 2% gel agarose was used for gel electrophoresis of PCR product.

4. Result

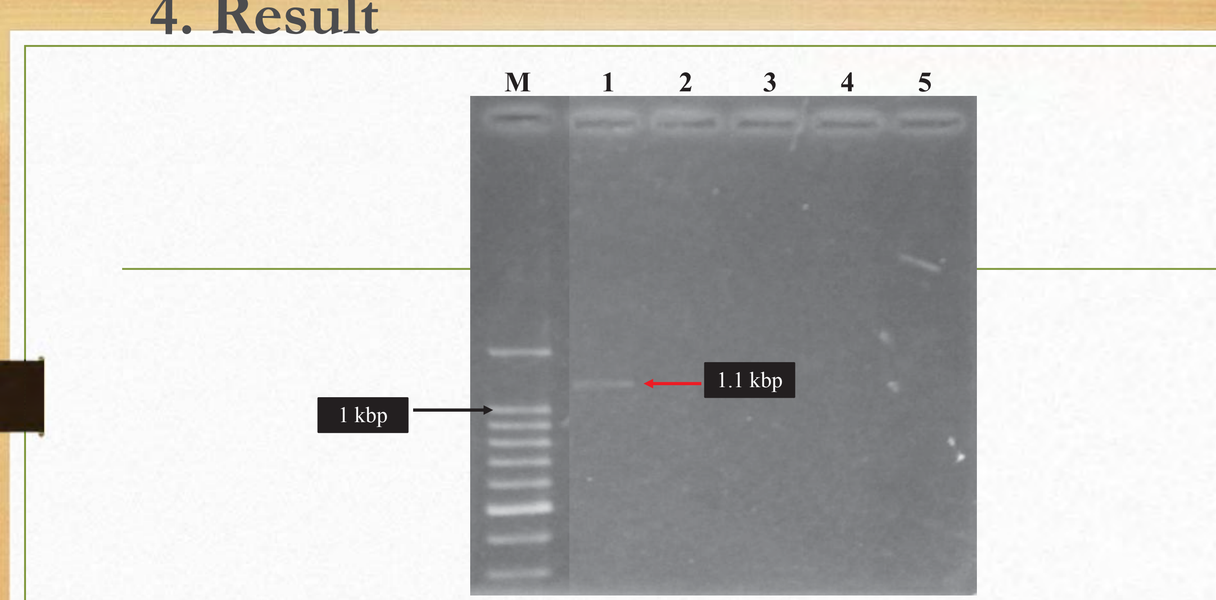


Fig 1. PCR assay of *Banana bunchy top virus* (BBTV) on banana aphid impregnated into FTA plant card with D11/D12 primers. The PCR band with the size of ~1.1kb (red arrow) was amplified from Sample 1 (lane 1). No band was amplified from Sample 2 and 3 (lanes 2-3). Lane 4 was negative control. The 100bp DNA ladder (Promega, USA) was included as marker.

5. Discussion

- The aphid sample collected BBTV infected banana plant (sample 1) resulted positive to BBTV presence. Sample 2 (aphids on BBTV-inoculated banana plant) and Sample 3 (aphids on BBTV-inoculated taro plant) gave negative to BBTV.
- The PCR assay for detecting BBTV on banana aphid can be performed with aphid-impregnated FTA plant card.

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Report 14. Method for developing phylogenetic tree using MEGA software

1. Lecturer

- Dr. Noriko Furuya

From DNA Data Bank of Japan (DDBJ).

2. Place and time

- Place: HOGOKEN meeting room
- Time: Nov. 26th, 2015

3. Contents

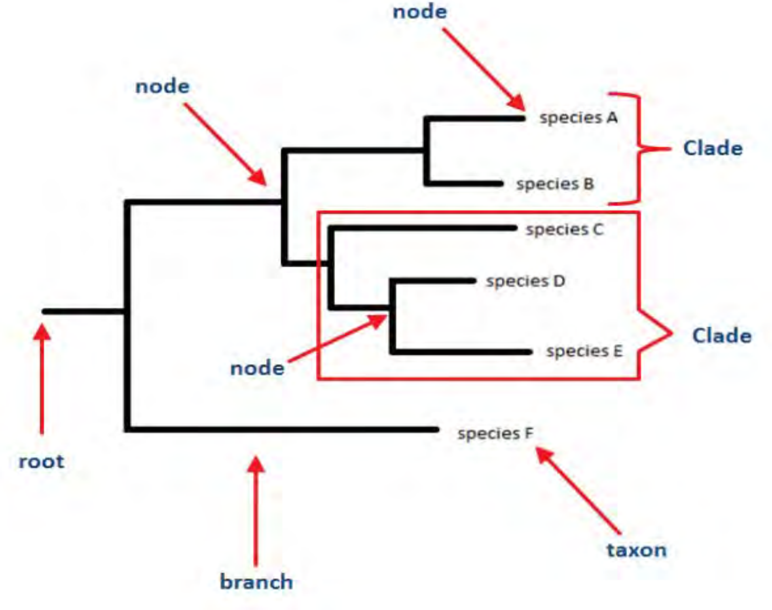
Source: http://www.dnabaser.com/articles/phylogenetic-tree/

3.1. Definition of Phylogenetic tree

- A branching diagram showing the inferred evolutionary relationships among various biological species based upon similarities and differences in their physical or genetic characteristics.

3.2. Main parts of a Phylogenetic tree

- Root
- Branches
- Nodes
- Leaves (taxon)



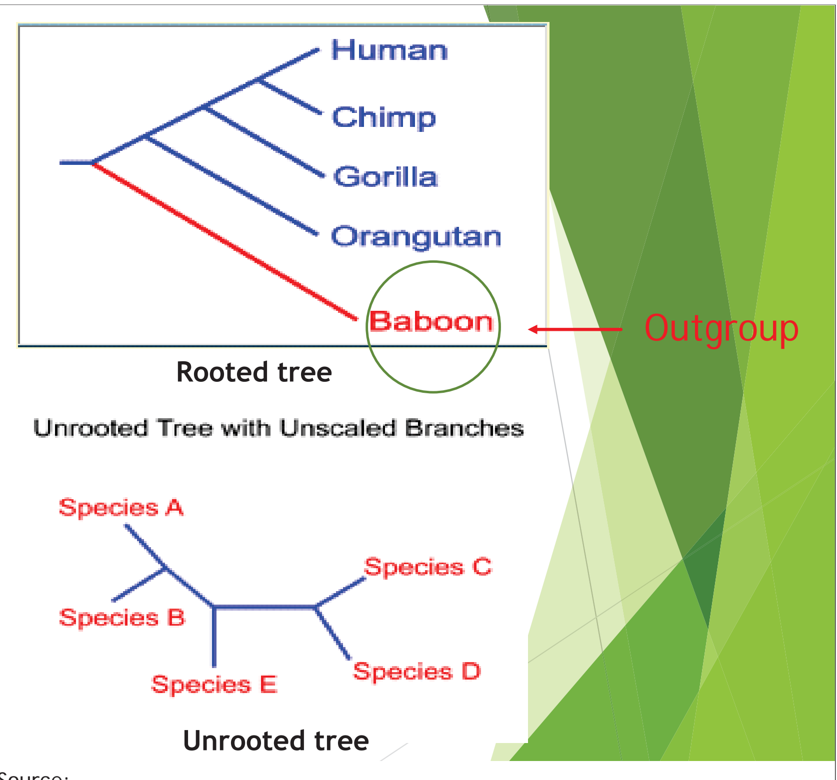
*Source:
http://www.cs.us.es/~fran/students/julian/phylogenetics/phylogenetics.html

3.3. Rooted tree

- A directed tree with an unique node corresponding to the most recent common ancestor of all the entries at the leaves of the tree.

- Outgroup is the entry that is not included in the target group at present.

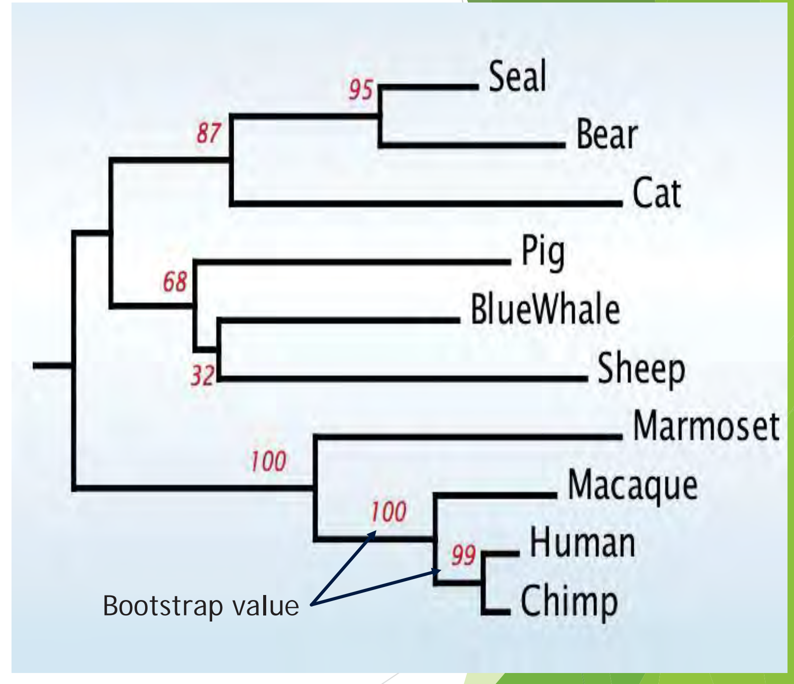
- If there is the outgroup entry, it is able to transform an unrooted tree to the rooted tree.



*Source:
http://www.ncbi.nlm.nih.gov/Class/NAWBIS/Modules/Phylogenetics/phylo9.html

3.4. Bootstrap method

Bootstrapping is a resampling analysis that involves taking columns of characters out of your analysis, rebuilding the tree, and testing if the same nodes are recovered. This is done through many (100 or 1000, quite often) iterations.



*Source: http://cabbagesofdoom.blogspot.jp/2013/04/how-to-read-phylogenetic-tree.html

3.5. MEGA software

- MEGA is an integrated tool for conducting sequence alignment, inferring phylogenetic trees, estimating divergence times, mining online databases, estimating rates of molecular evolution, inferring ancestral sequences, and testing evolutionary hypotheses.
- MEGA is used by biologists in a large number of laboratories for reconstructing the evolutionary histories of species and inferring the extent and nature of the selective forces shaping the evolution of genes and species

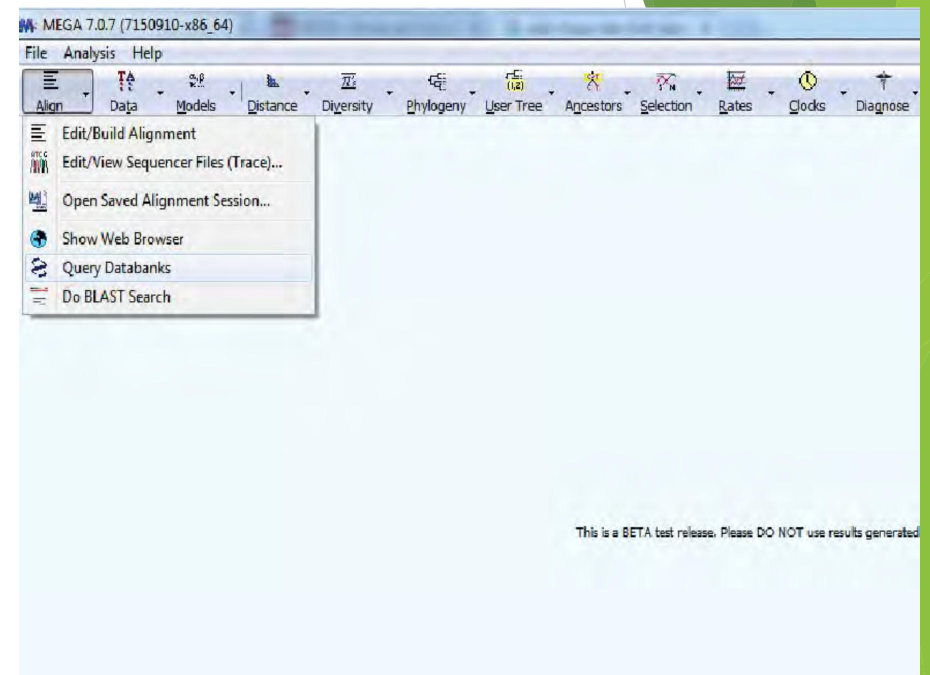
Source: http://www.megasoftware.net/

3.6. Method for constructing a phylogenetic tree using MEGA software

- Step 1.

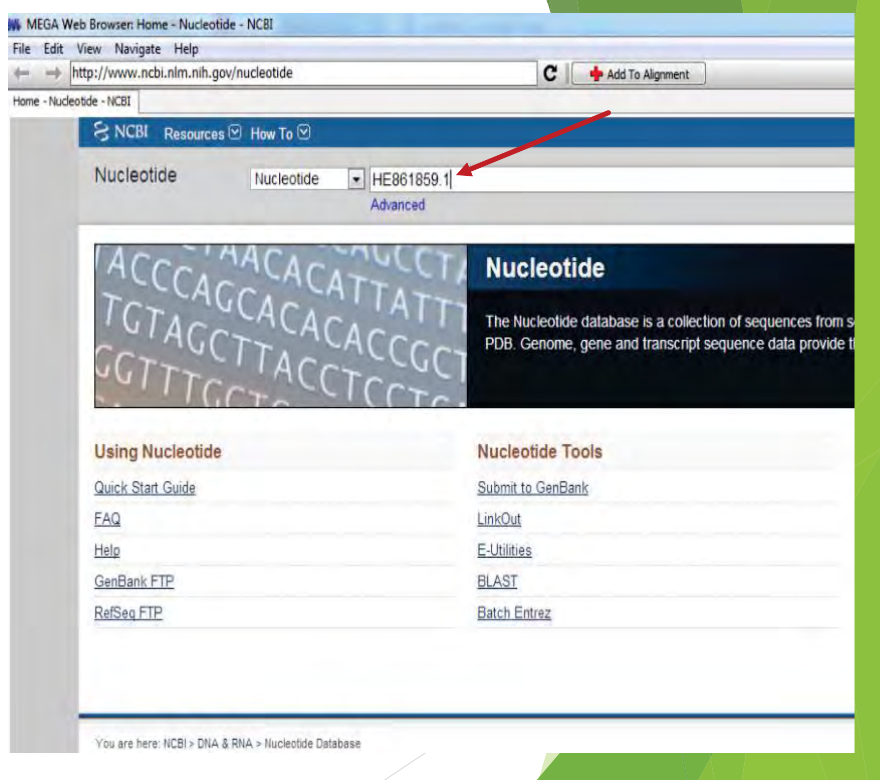
- Open MEGA software

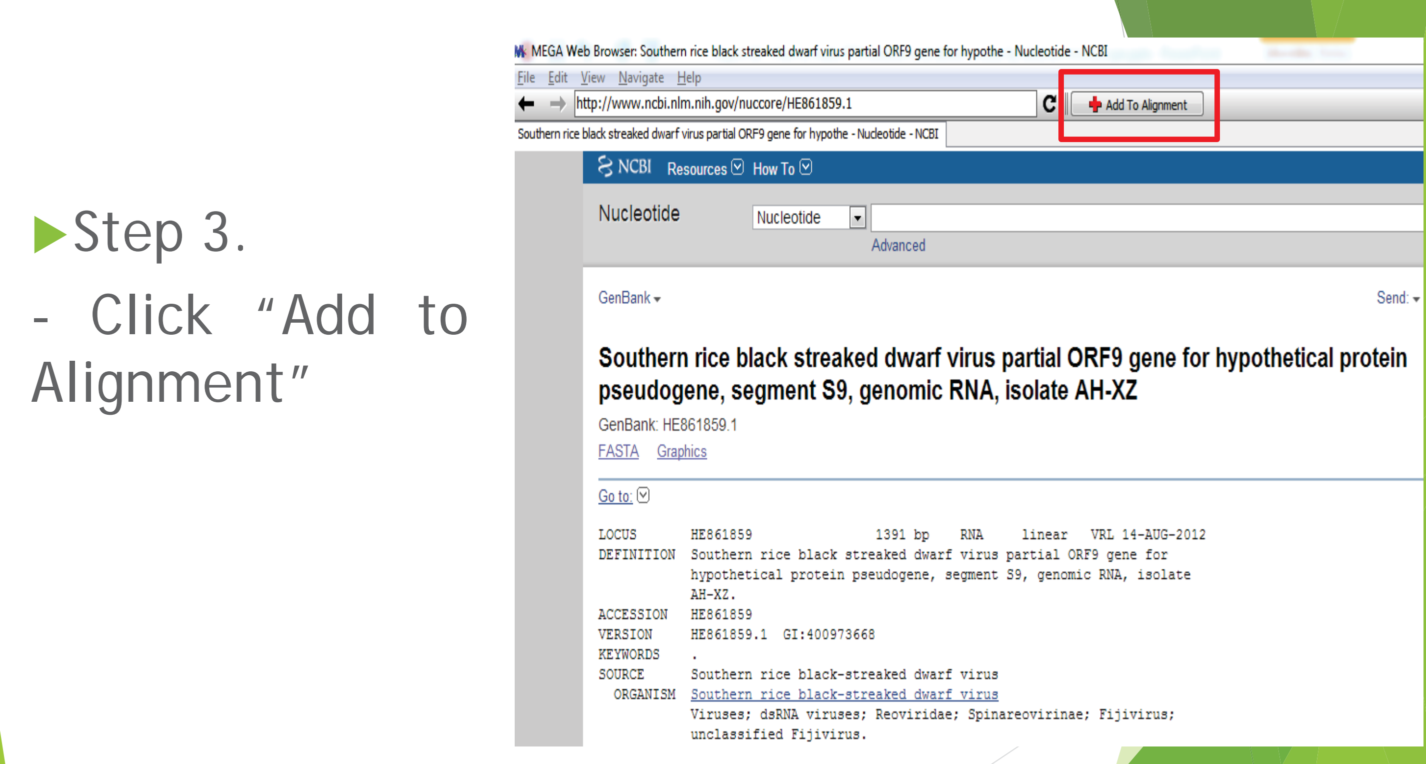
- Click "Align" and choose "Query Databanks"



Step 2.

- Type the accession of target nucleotide sequence.





Step 4.

- Finish the sequence label.
- Then, click "OK".

M7: Input Sequence Label

First word
S. rice black-streaked dwarf virus

Second word
AH-XZ

Third word

Fourth word

Use Initial for Genus Name

Full Information
Southern rice black streaked dwarf virus partial ORF9 gene for hypothetical protein pseudogene, segment S9, genomic RNA,

Sequence Label (manually editable)
S. rice black-streaked dwarf virus AH-XZ

OK Cancel

M7: Alignment Explorer

Data Edit Search Alignment Web Sequencer Display Help

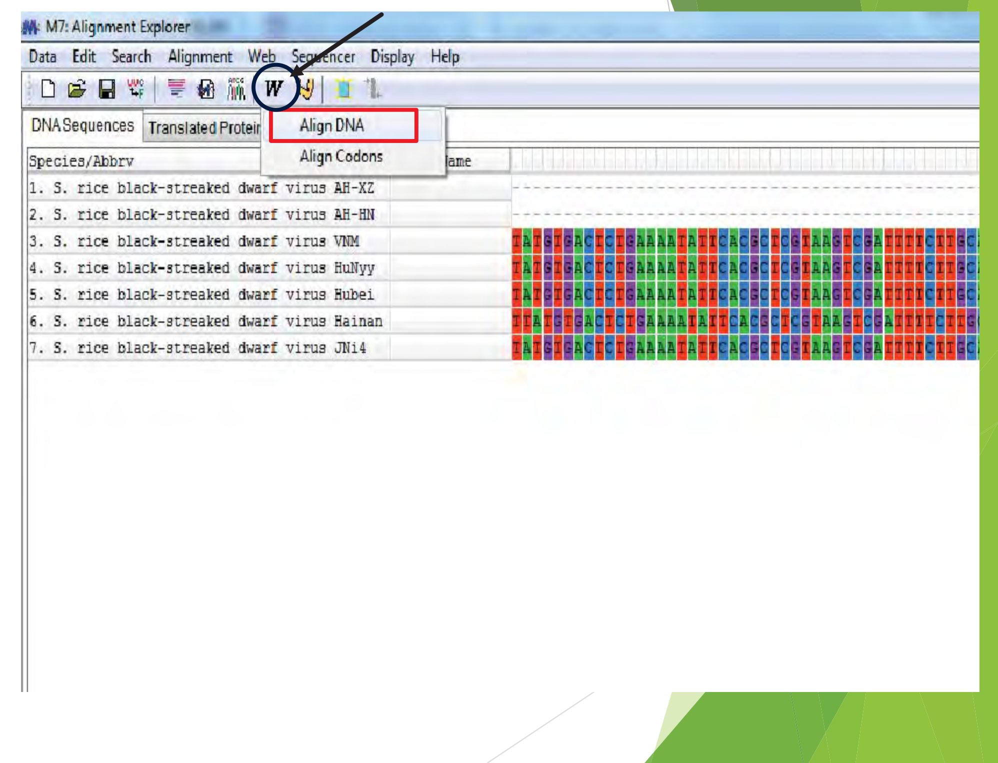
DNA Sequences Translated Protein Sequences

Species/Abbrv Group Name
1. S. rice black-streaked dwarf virus AH-XZ

Step 5.

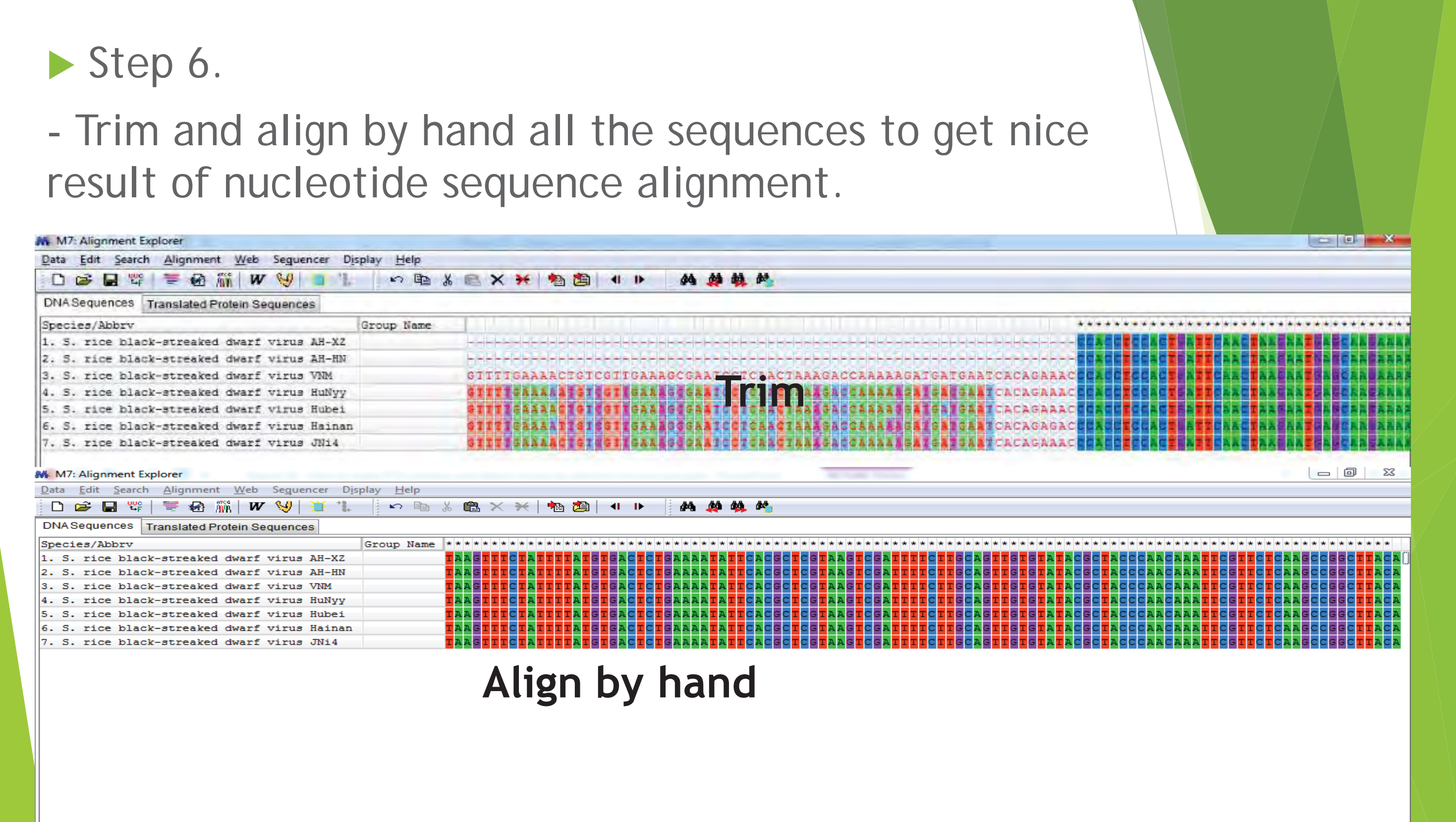
- Repeat 4 previous steps for each nucleotide sequence chosen to construct a phylogenetic tree.

- Afterwards, click icon "W" on the window of MEGA software. Press "Ctrl" + "A" to choose all nucleotide sequences and click "Align DNA"



Step 6.

- Trim and align by hand all the sequences to get nice result of nucleotide sequence alignment.



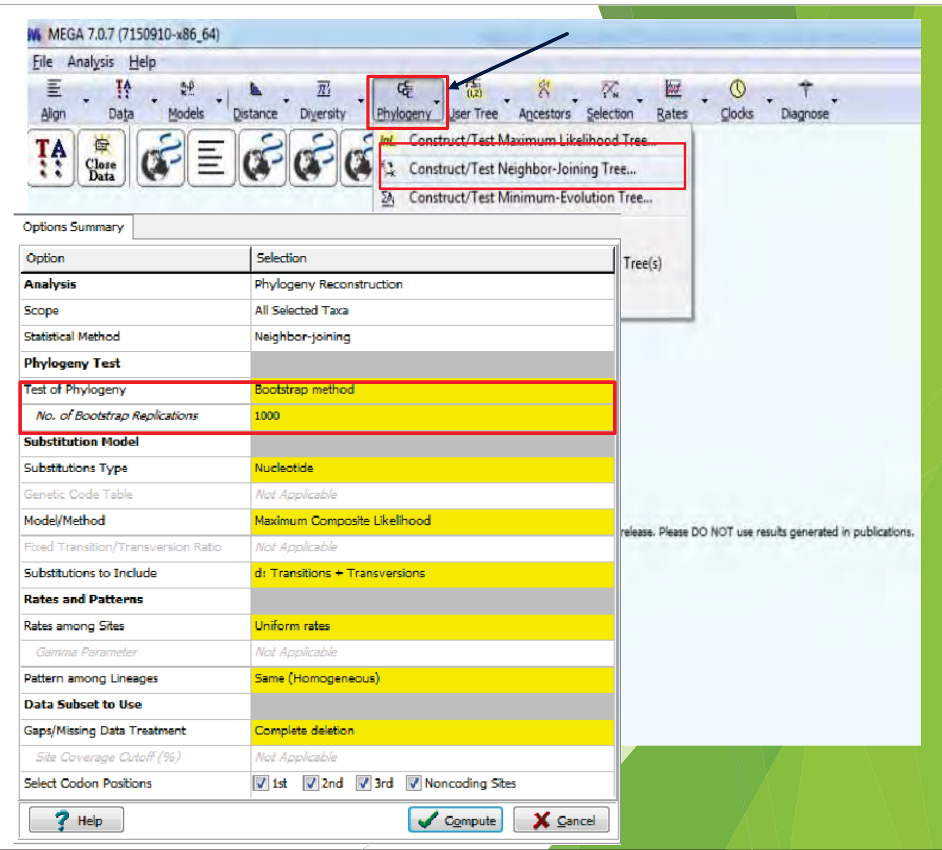
▶ Step 7.

- Click “Data” and choose “Phylogenetic analysis”

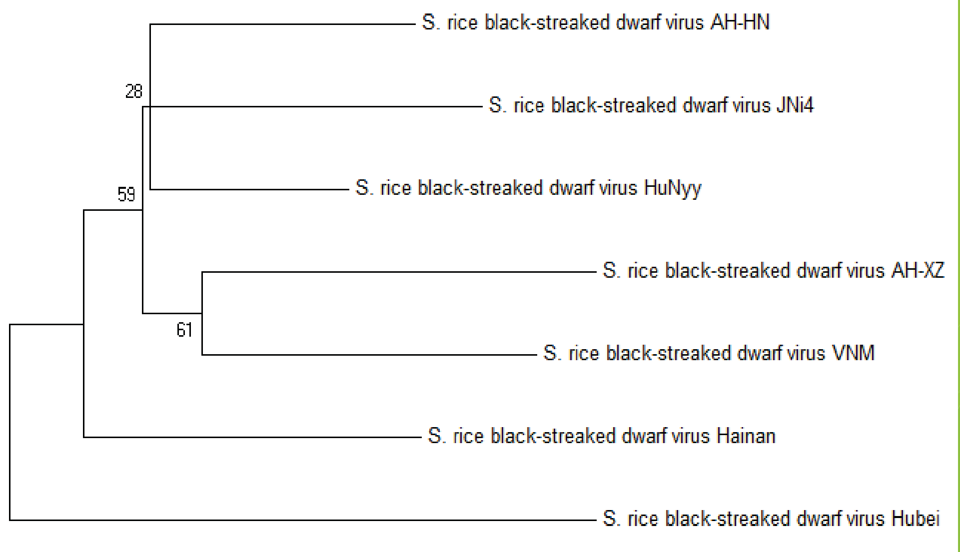
▶ Step 8.

- On the main window MEGA software, click “Phylogeny” and choose “”Construct/Test Neighbor-Joining Tree”.

- In option “Test of Phylogeny”, click “Bootstrap method” with “Bootstrap replication of 1000”. Click “compute”.



The phylogenetic tree will appear with bootstrap value.



Phylogenetic tree based on complete S9 nucleotide sequence of seven-isolates of SRBSDV

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Viet Nam

Report 15. Method for purification, ligation, transformation, cloning and sequencing of DNA of plant viruses.

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Nov. 18th - 27th, 2015

2. Samples and materials

- *2.1. For gel extraction*

- Wizard SV Gel and PCR clean-Up system (Promega, USA).

- *2.2. For Ligation*

- LigaFast™ Rapid DNA Ligation System (Promega, USA).

The LigaFast™ Rapid DNA Ligation System (Promega, USA)
1. pGEM-T vector; 2. 2X rapid ligation buffer; 3. T4 DNA ligase

The Wizard SV Gel and PCR clean-Up system (Promega, USA)
1. Membrane Binding Solution; 2. Membrane Wash Solution; 3. Nuclease-Free Water; 4. Minicolumn; 5. Collection Tube

2. Samples and materials

- *2.3. For transformation*

- pGEM-T vector-inserted DNA

- DNA fragments of *Banana Bunchy Top Virus* (BBTV) have been inserted into pGEM-T vector by T4 ligase.

- *E. coli* plasmid

- *2.4. For sequencing*

- LaboPass Plasmid Mini Kit/ Plasmid DNA purification Kit (COSMO GENETECH).

3. Protocols

3.1. Gel extraction

- Step 1: Gel electrophoresis

- Separate the DNA amplified products on a 2% agarose gel (if the size of expected DNA is smaller than 1.5kbp; on the other hand, it is recommended to used 1% agarose gel with 1kbp DNA ladder if the expected DNA size is larger than 1.5 kbp) in 1X TAE buffer and stain in Ethidium bromide solution.

- Load 10 µl 100bp DNA ladder and 12 µl solution (10 µl PCR product + 2 µl blue juice (loading dye)) into each well.

- Run the gel with 100 Voltage for 25-30 minutes.

- Prepare a clean 1.5 ml tube and get its weight.

- View the DNA band under UV light, use the blade to cut the gel and place in the pre-weighted 1.5 ml tube.

- Weight the tube again. *Weight of the gel = weight of the tube with gel - weight of the gel*

3.1. Gel extraction

- Step 2: Dissolving the gel

- Add 10 µl Membrane Binding Solution per 10 mg of gel slice.

- Vortex and incubate at 55°C for 10-15 minutes until gel slice is completely dissolved.

3.1. Gel extraction

- Step 3: Binding DNA

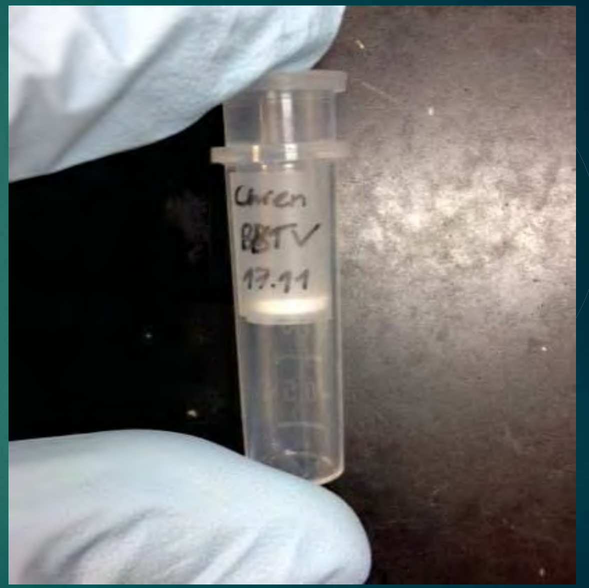
- Add 700 µl Membrane Wash Solution (Ethanol added).

- Transfer all dissolved gel solution into the minicolumn assembly.

- Incubate at room temperature for 1 minute.

- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the solution and re-insert minicolumn into collection tube.



3.1. Gel extraction

- Step 4: Washing

- Insert Minicolumn into Collection tube.

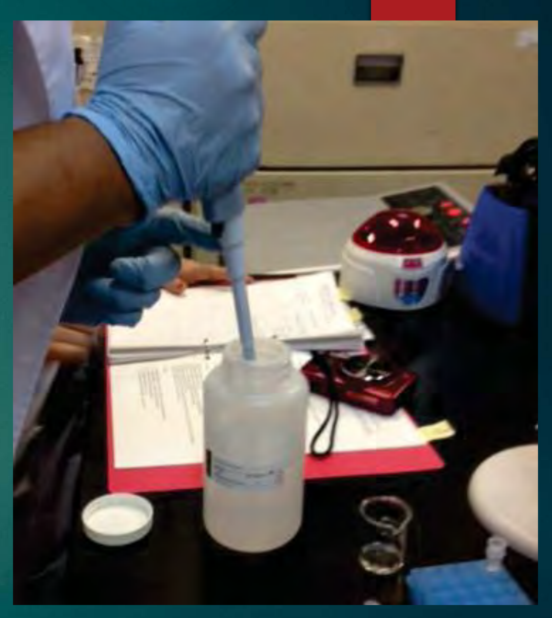
- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the solution and re-insert minicolumn into collection tube.

- Repeat step 1 with 500 µl Membrane Wash Solution.

- Centrifuge the minicolumn assembly at 16,000 rpm (KUBOTA 3300) for 5 minute.

- Empty the collection tube and re-centrifuge the minicolumn assembly for 1 minute with opening the microcentrifuge lid to alow evaporation of any residual ethanol.



3.1. Gel extraction

- Step 5: Elution

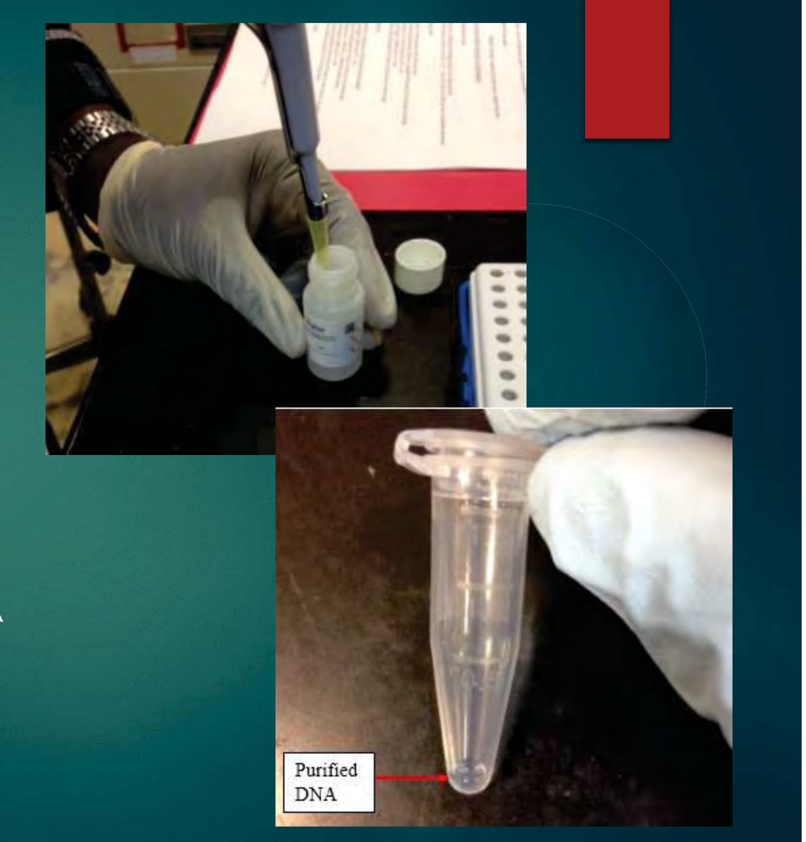
- Carefully transfer minucolumn to new 1.5 ml tube.

- Add 30 µl Nuclease-Free Water to the minicolumn.

- Incubate at room temperature for 1 minute.

- Centrifuge at 16,000 rpm (KUBOTA 3300) for 1 minute.

- Discard the minicolumn and store DNA at 4°C or -20°C.



3.2. Ligation

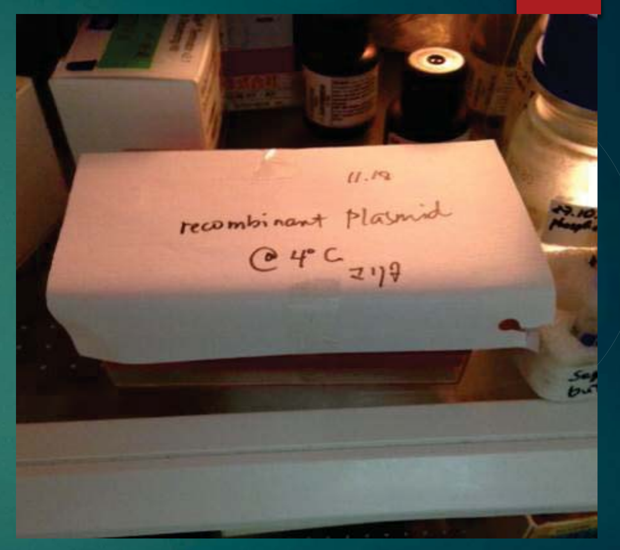
- Step 1: Add the cocktail mixture into 1.5 ml tube following:

2X Rapid ligation buffer	5 µl
pGEM-T vector	1 µl
T4 DNA ligase	1 µl
Purified DNA	3 µl
Total	**10 µl**

3.2. Ligation

- Step 2: Mix well with pipette and incubate over night at 4°C (Sharp SJ56S).

- After ligation, the insert DNA is will be transformed into bacterial cells for propagation for sequencing



3.3. Transformation of inserted DNA into E. coli plasmid

- Step 1.

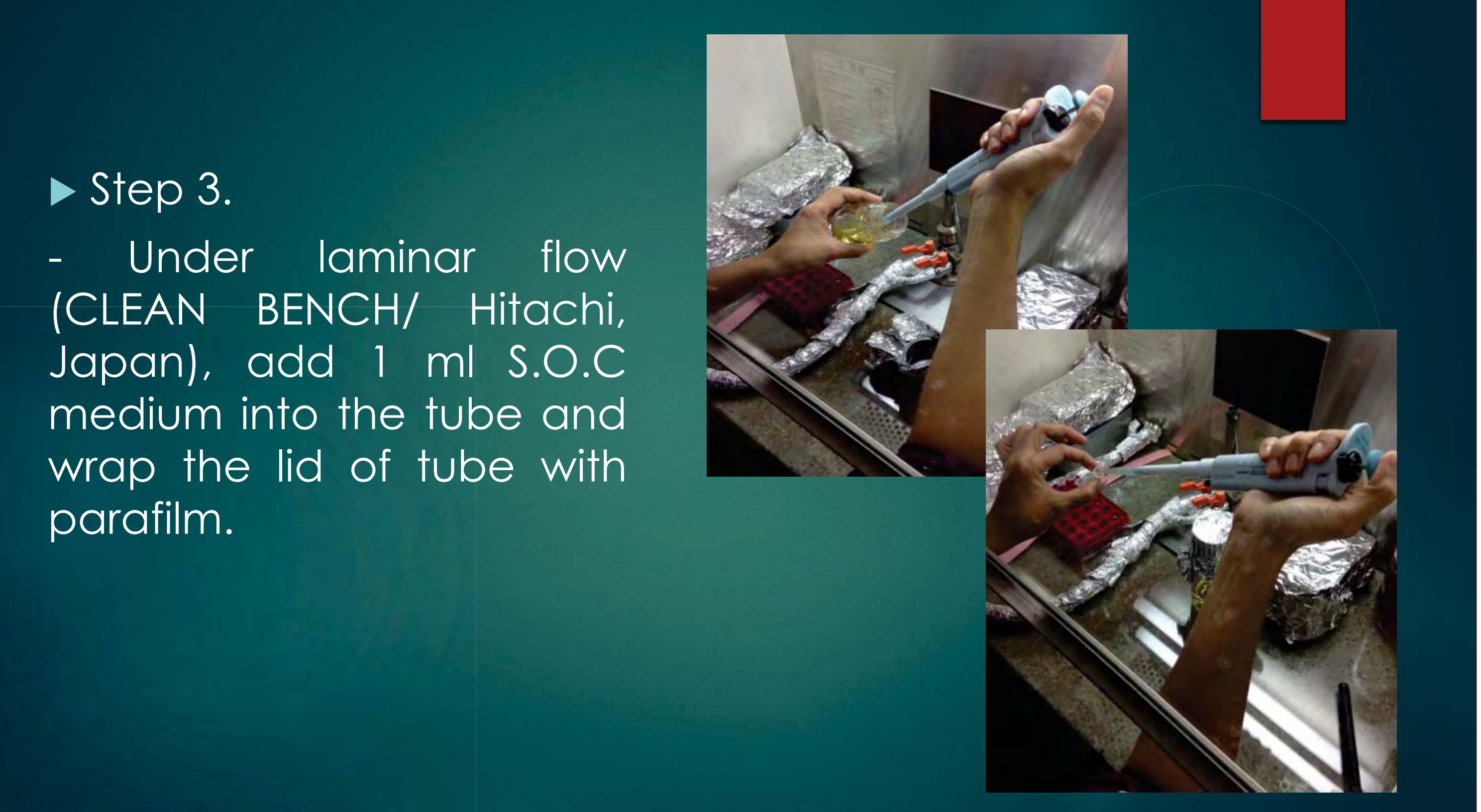
- Leave the competent *E. coli* (105 µl/ tube) in ice box for 1.0 – 1.5 hours. Using pipette to mix gently the competent *E. coli* to be totally dissolved.

3.3. Transformation of inserted DNA into E. coli plasmid

- Step 2.

- Add 50 µl competent *E. coli* to inserted DNA and incubate in ice box for 30 minutes.

- Put the tube including competent *E. coli* and inserted DNA in heat block at 42°C (Dry Thermo Unit) for 45 seconds and immediately transfer back to ice box for 2 minutes.

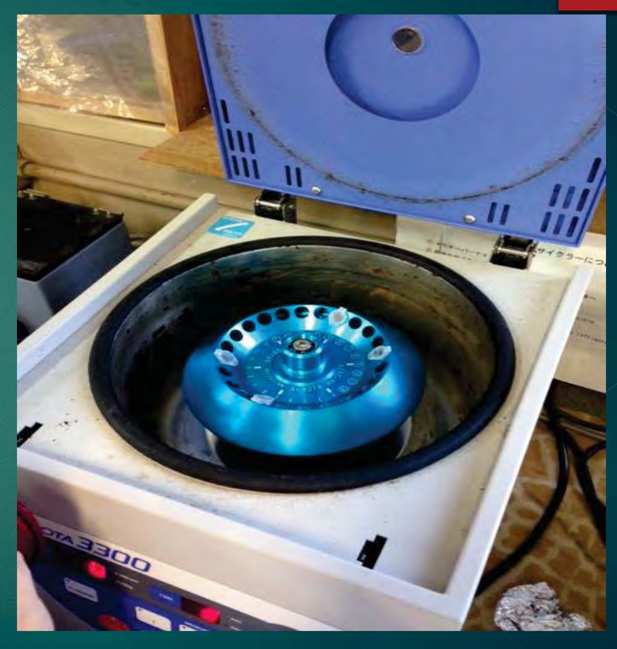


▶ Step 4.

- Shake the tube horizontally by shaker (Bio Shaker BR-15LF/ TAITEC) for 45 minutes at 37°C.

▶ Step 6.

- After shaking, centrifuge with 15,000 rpm (KUBOTA 3300) for 2 minutes to get pellet of *E. coli*.



- Step 6.

- Use the pipette to remove the S.O.C medium and just remain about 200 µl in tube.

- Step 7.

- In the laminar flow, drop the solution in tube onto the petri with LB medium and spread well with sterilized triangle rod until the solution completely dry.

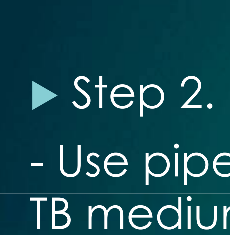
- Step 8.

- Wrap the petri with parafilm and incubate at 37°C overnight (AS ONE) and transfer to 4°C for 1-2 days (Sharp SJ56S).

3.4. Cloning recombinant plasmid in TB medium

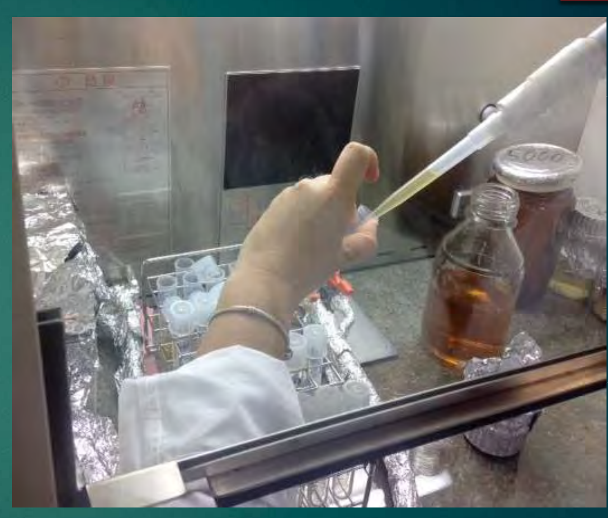
- Step 1.

- Select 05 white colonies of recombinant plasmid from the petri and 01 blue colony as negative control.



- Step 2.

- Use pipette to transfer 4 ml TB medium into one 15 ml falcon tube.



- Step 3.

- Use sterile toothpick to take a single colony and then transfer into falcon tube.

Step 4.

- Flame the used toothpick afterwards to avoid the contamination.

Step 5.

- Cover the lid of falcon tube with parafilm, then shake (Bio Shaker BR-15LF/ TAITEC) overnight at 37°C.

3.5. Miniprep

- Step 1.

- After shaking the recombinant plasmid overnight, take the falcon tube from shaking machine and remove the parafilm from lid of tube.

- Step 2.

- Centrifuge the falcon tube at 3,500 rpm (TOMY LC-100 Low speed centrifuge) for 7 minutes.

Step 2.

- Pour off the TB medium and keep the pellet.

Step 3.

- Add 250 µl S1 buffer with RNAse added before using (do not let the tip touch the tube).

- Re-suspend the pellet and vortex the falcon tube for 30 seconds and transfer all the suspension into new 1.5 ml tube.

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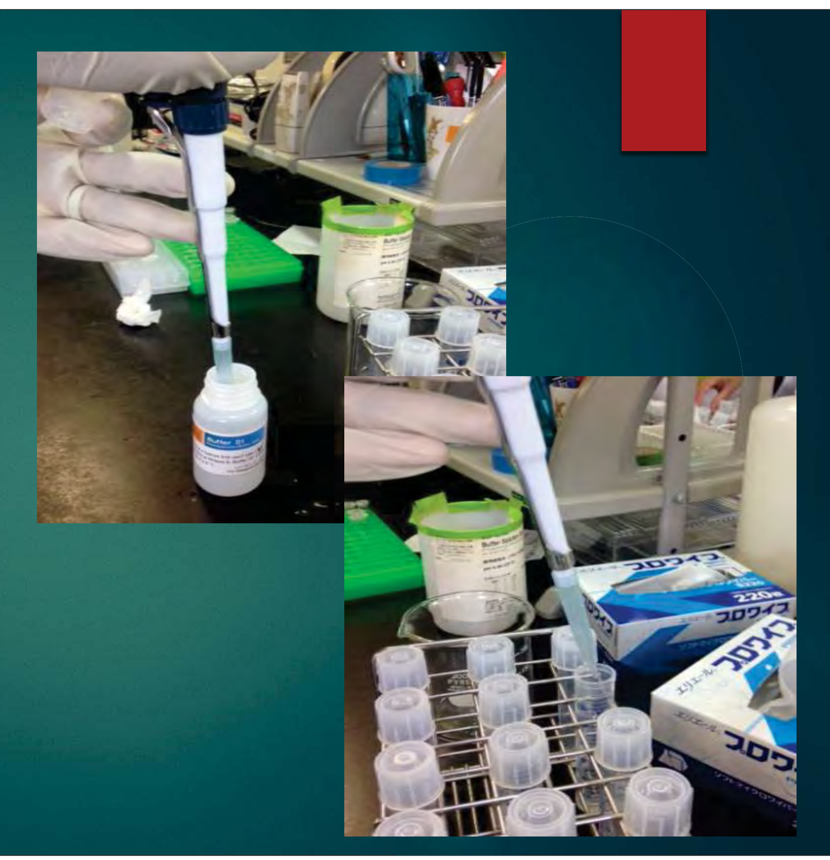
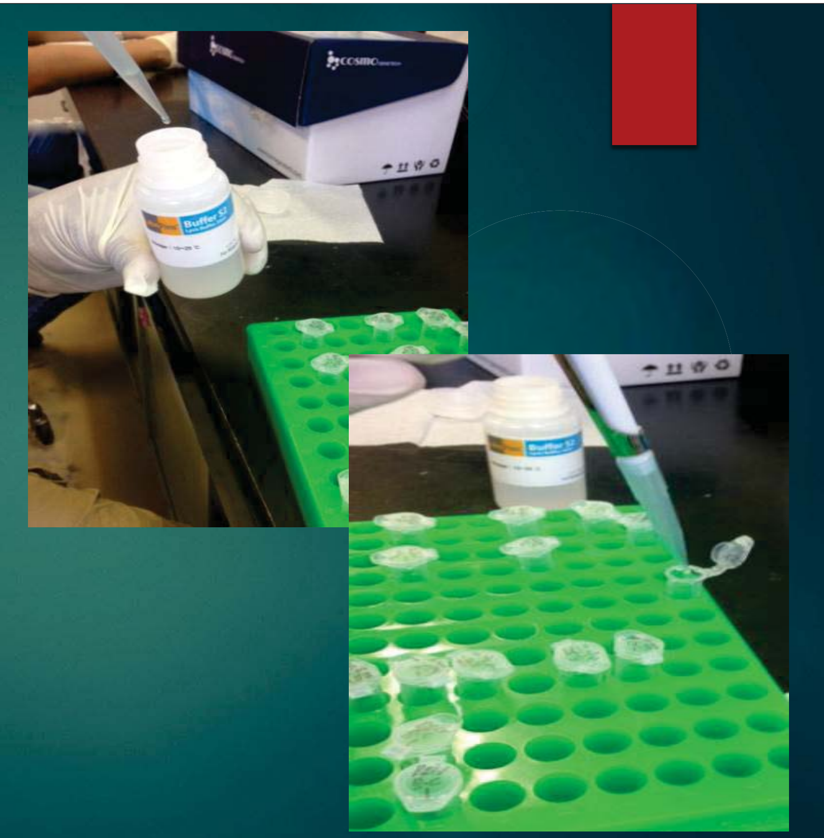


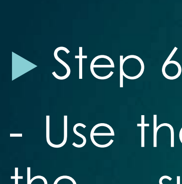
Step 4.

- Add 250 µl S2 buffer (S2 buffer must be shake before using) and afterwards invert the tubes 3 – 4 times (do not vortex the tubes).
- Incubate the tubes at room temperature for 5 minutes.



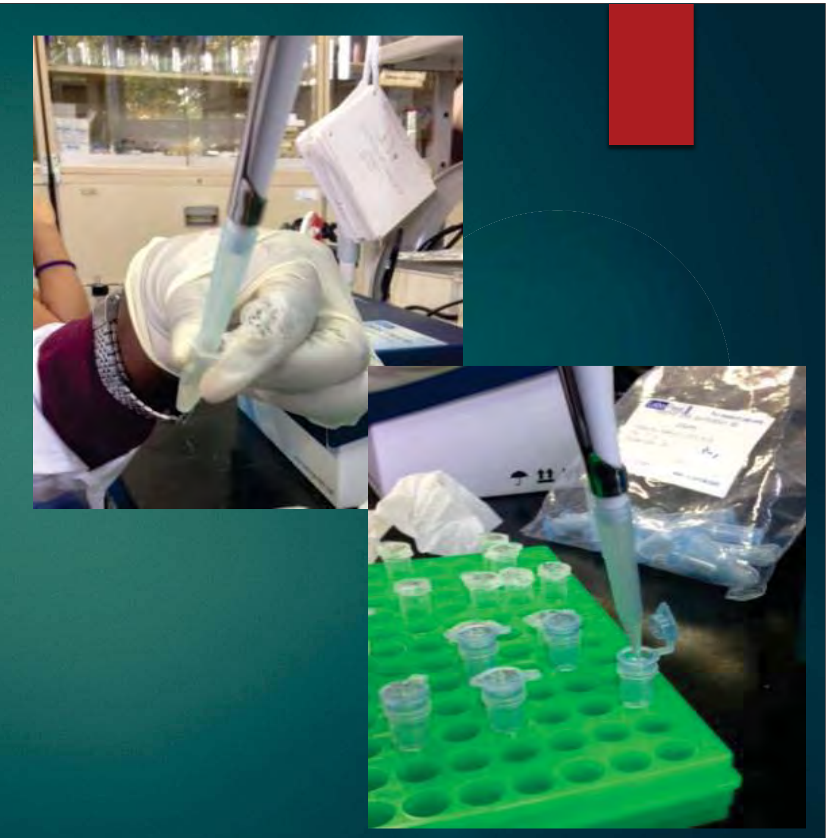
Step 5.

- Add 350 µl S3 buffer into the tubes (S3 buffer must be shake before using) and then invert the tube 3 – 4 times.
- Centrifuge the tubes for 10 minutes at 14,000 rpm (KUBOTA 3300).



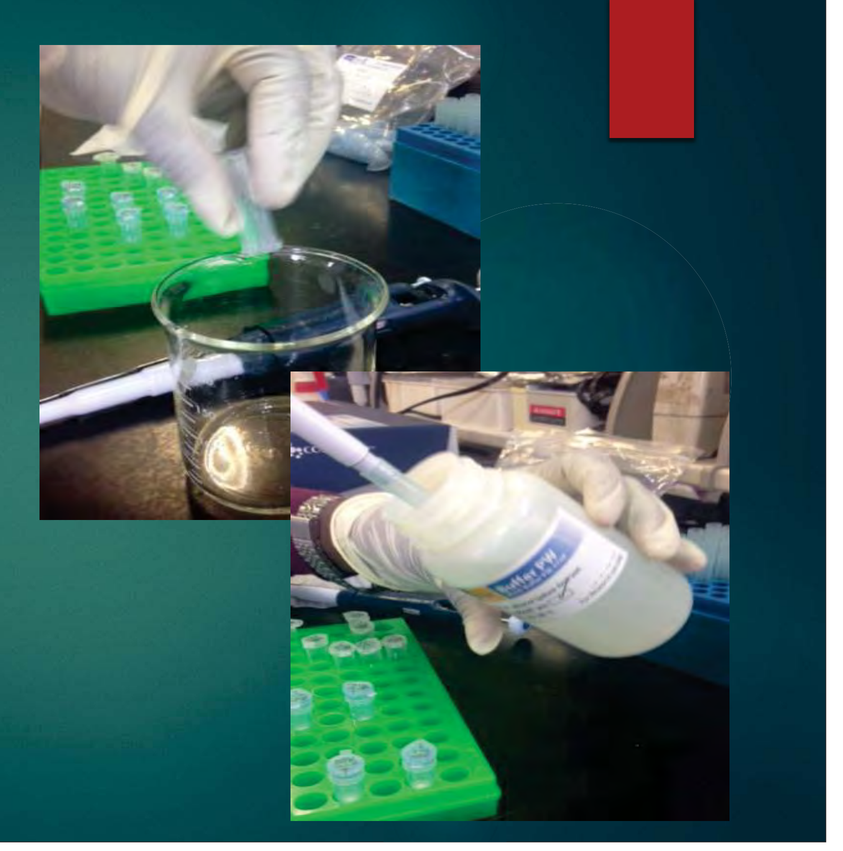
Step 6.

- Use the pipette to transfer the supernatant (clear solution) into the spin column. The plasmid will be trapped in the filter.
- Centrifuge the tubes for 10 minutes at 14,000 rpm (KUBOTA 3300).



Step 7.

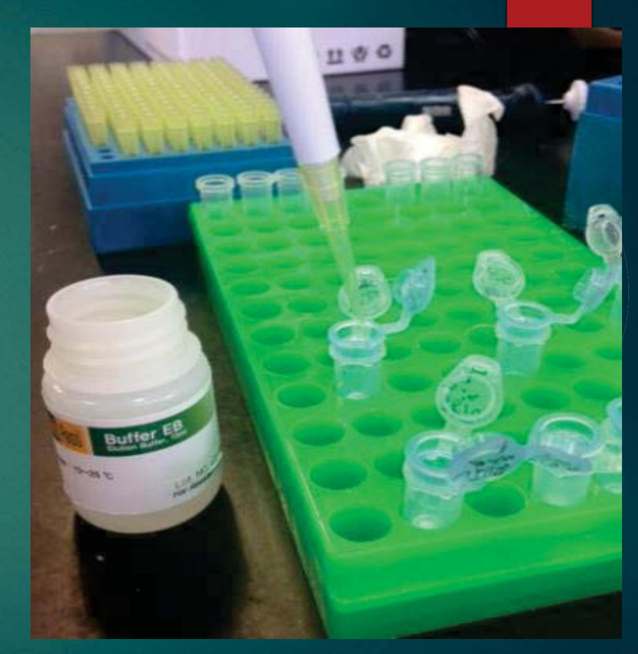
- Pour off the liquid, afterwards adding 750 µl PW buffer and centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300) (PW buffer will further wash the filter to purify the plasmid).



- Step 8.

- Pour off the liquid again and centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300). Afterward, transfer the spin column into new 1.5 ml tube.

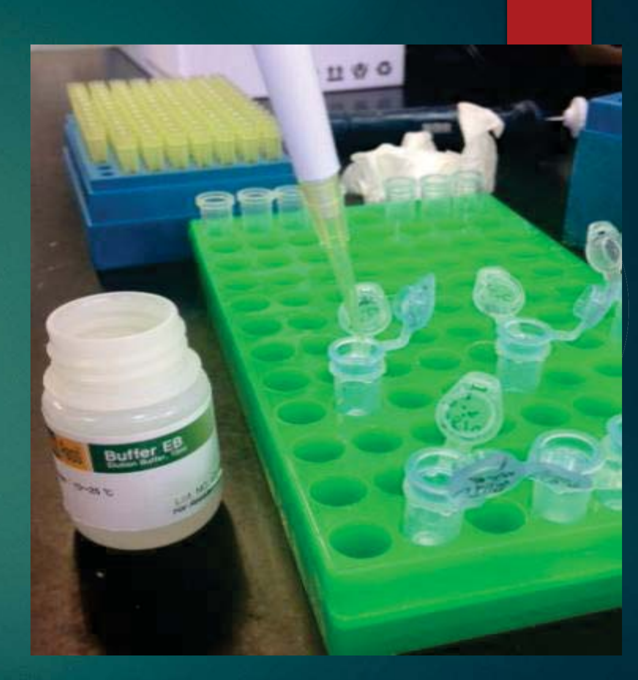
- Add 50 µl EB buffer into the center of the filter and keep at room temperature for 1 minute (EB buffer will dissolve the plasmid from the filter).



- Step 9.

- Centrifuge the tubes for 1 minutes at 14,000 rpm (KUBOTA 3300) and keep the plasmid.

- The collected plasmid is used in the next steps for the sequencing or kept at 4°C.



3.6. Insert check

- Prepare 1.5% agarose gel.
- Load 4µl 1.5 kb DNA ladder (Promega, USA) as a marker onto the first well of 1.5% prepared agarose gel.
- Mix 2µl loading buffer (6X load dye) and 2µl DNA plasmid of blue colony and load onto the agarose gel as a negative control.
- Mix 2µl loading buffer and 2µl DNA plasmid of each samples and load onto agarose gel.
- Run samples for 25 – 30 minutes using electrophoresis machine (Mupid-2plus/ Advance).
- After running, stain the gel by submerging it into Ethidium bromide solution for 3 minutes.
- De-stain the gel in distilled water for 1 minute.
- View DNA plasmid band under UV illumination and take photo using EDAS 290 (Kodak, Japan).

3.7. Cycle Sequence

- Step 1:

- Prepare the cocktail mixture; calculate the required amount as follow:

q.s. (double distilled water)	2.0 µl
Sequence buffer (Big Dye Terminator 5X sequencing buffer)	1.0 µl
*Primer (sp6/ T7)	0.5 µl
Premix	2.0 µl
Inserted plasmid DNA (PCR product)	4.5 µl
Total	**10 µl**

note: 1 sample of successfully inserted DNA will be conducted cycle sequence with 2 reactions separately (one with sp6 primer and one with T7 primer).

- Step 2:

- Conduct Cycle sequence by PCR machine (DNA Engine/ BioRAD) with the following PCR conditions:

Temperature	Time	Cycles
96°C	10 seconds	24
50°C	5 seconds	
60°C	4 mintes	
4°C	endless	

3.8. Precipitation

- Step 1:

- After thermal cycle, prepare the cocktail mixture in 1.5 ml tube as below:

Cycle sequencing product	10.0 µl
3M Acetic acid	1.0 µl
99.5% Ethanol	30.0 µl
Total	41 µl

3.8. Precipitation

- Mix and centrifuge the cocktail mixture briefly (KUBOTA 3300).
- Put the 1.5 ml tube in ice box for 10 minutes.
- Centrifuge at 20°C for 20 minutes at 14.000 rpm (HITACHI, Japan).
- Discard the supernatant and keep the pellet.
- Add 100 µl 99.5% ethanol (not cold) into the tube, afterwards centrifuging for 5 minutes at 14.000 rpm (KUBOTA 3300).
- Discard the supernatant and keep the pellet.
- Add 100 µl 99.5% ethanol (kept in -30°C) into the tube, then roll (horizontal) the tube for 20 – 30 seconds.
- Centrifuge the tube for 5 minutes at 14.000 rpm (KUBOTA 3300).
- Discard the supernatant and dry pellet for 2 minutes at room temperature with lip of tube is opened.
- Put the tube in heat block (Dry Thermo Unit) at 95°C for 2 minutes with lip of tube is opened.
- Take the tube from heat block and put it again in ice box for 5 minutes with lip of tube is closed.
- Keep the pellet at 4°C (Sharp SJ56S).

3.9. DNA analysis using Automate Sequencer

- Step 1.

- Dissolve the pellet with 20 µl Hi-Di formamide.

- Transfer the solution into the 96-Well Reaction Plate carefully.

Step 2.

- Check all wells to ensure that there are no bubbles inside the wells.

- Cover the plate by Plate Septa 96-Well.

- Keep the plate 4°C (Sharp SJ56S) for sequencing using sequencer machine.

THANK YOU FOR YOUR ATTENTION

Terraced Field in Sapa !

Viet Nam

Annex 16

Report 16. Method for extraction of dsRNA from double-stranded RNA plant viruses

1. Lecturer

Ph.D. **Tomohide Natsuaki**

Vice-President for International Exchange

Vice-Dean for Research & Professor

Utsunomiya University

2. Place and time

- Place: Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University
- Time: Dec. 3rd – 4th, 2015

3. Samples and dsRNA target

- **3.1. Sample:**

Japanese snake gourd (*Trichosanthes pilosa*) infected by *Cucumber mosaic virus* (CMV) is stored at - 80°C.

- **3.2. dsRNA target**

dsRNA from *Cucumber mosaic virus* (CMV)

4. Protocols

- Step 1.

- Weigh 05 grams of sample and grind well using cold mortal & pestle

4. Protocols

- Step 2.

- Add 10 ml 2X STE buffer and 50 µl 2-mercaptoethanol into mortal.

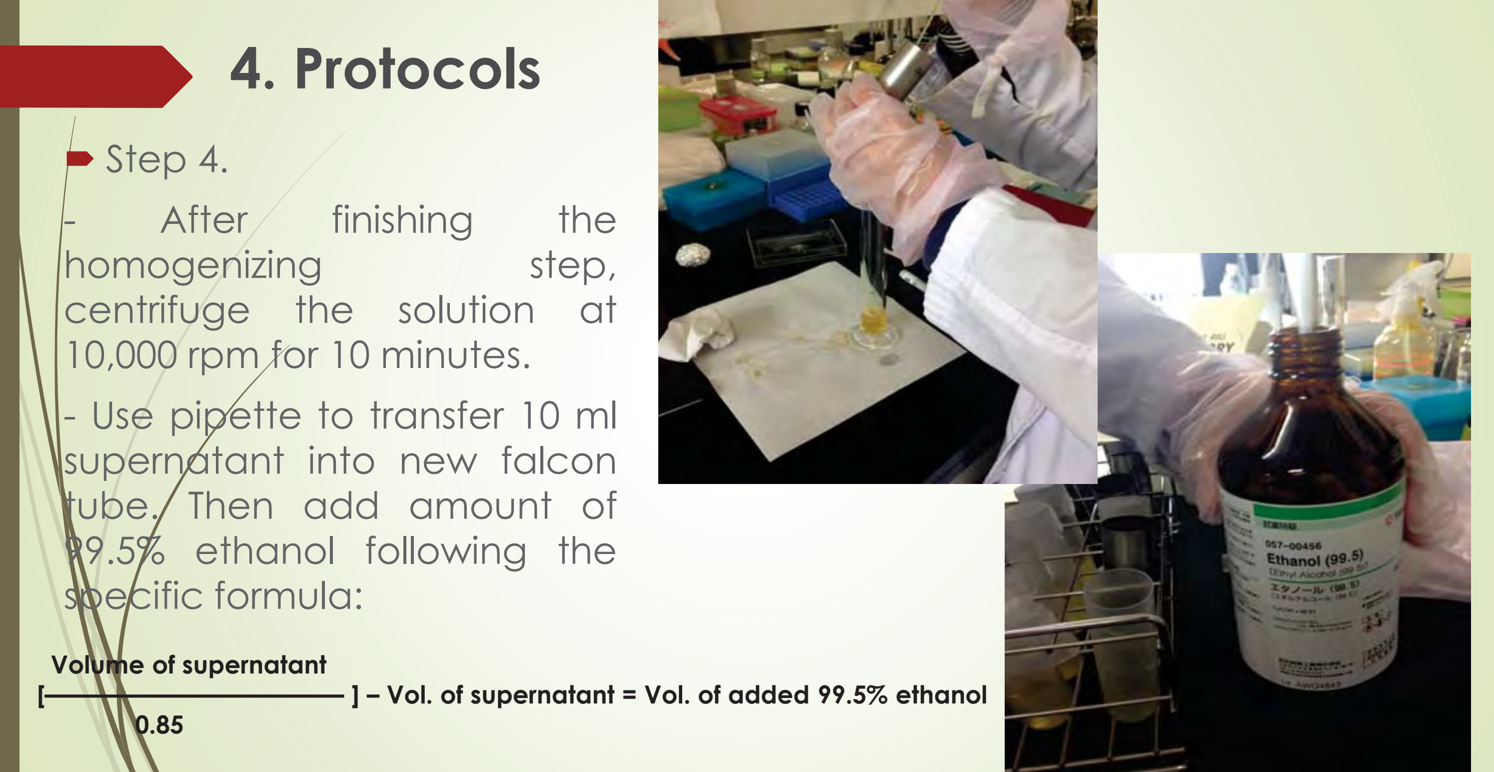
- Mix with sample, then transfer the plant sap into the homogenizer tube.

4. Protocols

- Step 3.

- Keep the mortal with remain of plant sap, add 2.5 ml phenol; 2.5 ml chloroform and 0.1 ml iso amyl alcohol into the mortal, then mix well.

- Transfer the solution into the same homogenizer tube (step 2) and homogenize at 5000 rpm for 5 minutes (Ace Homogenizer, Nihonseiki Kaisha ltd.).



4. Protocols

- Step 4.

- After finishing the homogenizing step, centrifuge the solution at 10,000 rpm for 10 minutes.

- Use pipette to transfer 10 ml supernatant into new falcon tube. Then add amount of 99.5% ethanol following the specific formula:

$$\left[\frac{\text{Volume of supernatant}}{0.85}\right] - \text{Vol. of supernatant} = \text{Vol. of added 99.5\% ethanol}$$

4. Protocols

- Step 5.

- Incubate the solution at 4°C for 60 minutes, then centrifuge at 10,000 rpm for 10 minutes.

- Use pipette to transfer all the supernatant into new glass tube. Afterwards, add 1 g fibrous cellulose powder (Whatman) into glass tube.

- Vortex the mixture for 15 minutes with 30 seconds interval.

4. Protocols

- Step 6.

- Cut the tissue paper to make a filter and push it inside the chromatography tube.

- Hang up and transfer the mixture into the chromatography tube.

- Place the glass under chromatography tube.

4. Protocols

- Step 7.

- Transfer 100 ml 1X STE buffer and 15% ethanol into conical flask.

- Hang it up over the chromatography tube. Use the specific pipe to connect conical flask and chromatography tube.

- Let the sample precipitate totally, put again the filtered solution into the chromatography tube. The total DNA will be kept in cellulose powder.

4. Protocols

- Step 8.

- Weigh 0.8 g fibrous cellulose powder (Whatman) and put it into new glass tube. Afterwards, place the glass tube under the chromatography tube.

- Add 20 ml 1X STE buffer into the chromatography tube and let the solution precipitate totally.

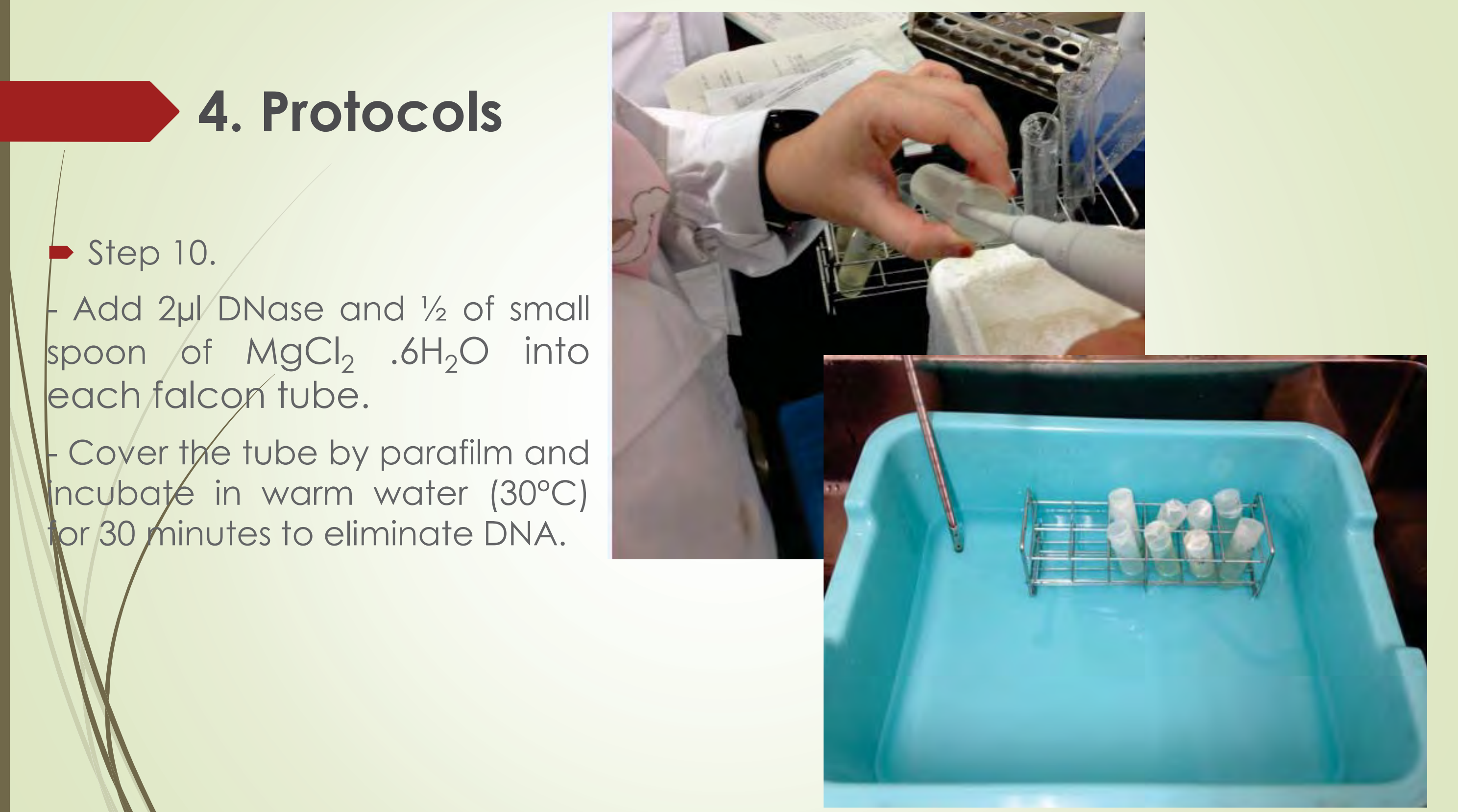
4. Protocols

- Step 9.

- Add 3.6 ml 99.5% ethanol into the new cellulose solution and Vortex for 15 minutes with 30 seconds interval.

- Put the mixture into the chromatography tube and let the solution precipitate totally.

- Add 8 ml 1X STE buffer into the chromatography tube and let it filter down into a new falcon tube. Repeat the same process for another falcon tube. (Total volume of filtered solution is 16 ml (2 falcon tube)).



4. Protocols

- Step 11.

- Add 8 ml 99.5% ethanol into each falcon tube with DNA eliminated solution.

- Store at -20°C overnight or -80°C for 1 hour.

4. Protocols

- Step 12.

- Centrifuge the sample in cold temperature (0°C) at 10,000 rpm for 15 minutes.

- Discard supernatant and keep the pellet.

- Prepare the RNA-dissolving solution by mixing 100 µl 10X loading dye and 500 µl RNase free water

- Put the falcon tube upside down inside the dryer for 5 minutes to remove ethanol totally.

4. Protocols

- Step 13.

- Use pipette to drop 50 µl RNA-dissolving solution into each tube and mix well by laboratory spatula to dissolve RNA.

- Spin the tube for few seconds and transfer all the sample into 1 PCR tube.

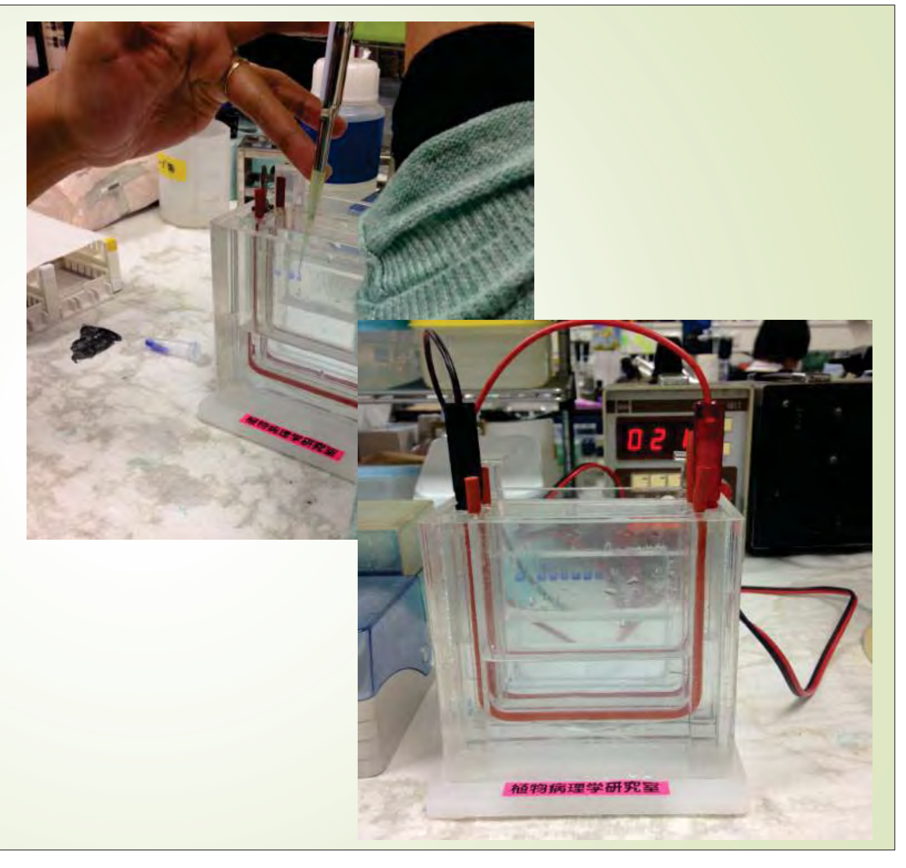
4. Protocols

- Step 14.

- Pour 5% polyacrymide gel into the gel case and place under the light to fasten the solidification of gel.

- Put 1X TBE buffer inside gel case. Make sure that middle part of gel case is filled up by 1X TBE buffer.

- Load 10 µl 1kb DNA ladder as a marker and 6 µl sample into the gel. Conduct gel electrophoresis at 20 mA for 50 minutes.



4. Protocols

- Step 15.

- After finishing gel electrophoresis, stain gel with Ethidium bromide for 1 minute and wash with dH_2O for 30 seconds.

- View the result by UV transillumilnator.

dsRNA 1
dsRNA 2
dsRNA 3
dsRNA 4

THANK YOU FOR YOUR ATTENTION

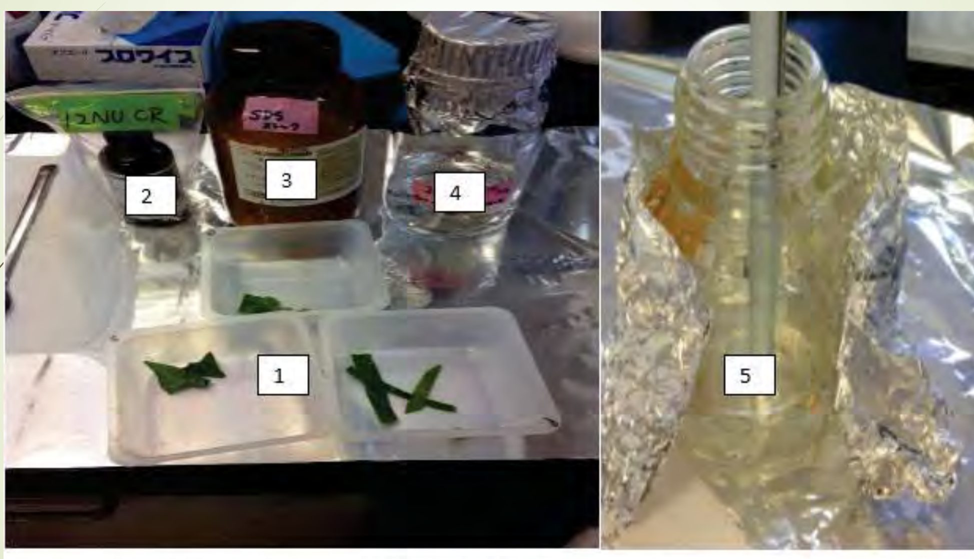
Viet Nam

Report 17. Method for extraction of RNA of plant viruses using phenol-chloroform solution

1. Place and time

- Place: Laboratory of Plant Pathology, Faculty of Agriculture, Utsunomiya University
- Time: Dec. 7th, 2015

2. Samples and materials



1. Samples (potyvirus-inoculated passionfruit plant); 2. 2-mercaptoethanol; 3. SDS; 4. 2X STE buffer; 5. Phenol-Chloroform solution

3. Protocols

- Step 1.

\- Make the extraction buffer:

2X STE buffer .... 1 ml
SDS.......................................0.01 g
2-mercaptoethanol...............10µl

Mix well by vortex.
This volume is used for extraction of 1 sample.

3. Protocols

- Step 2.

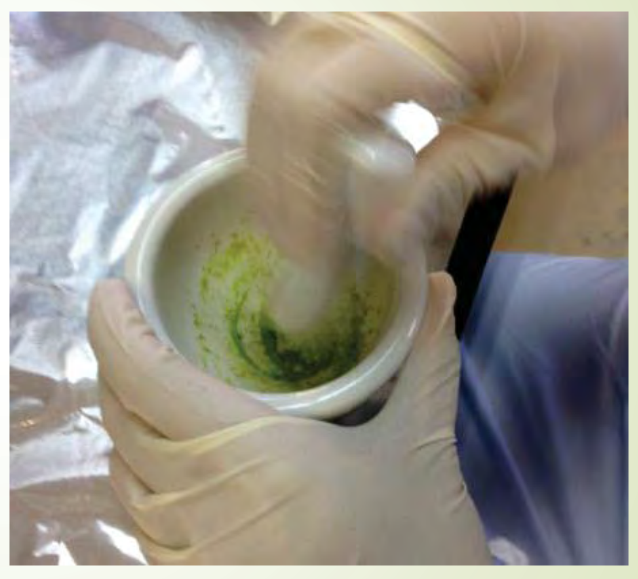
- Weigh 0.1g sample and put it into the cold mortar and pestle.

- Add liquid nitrogen into mortar and grind well.

3. Protocols

- Step 3.

- Add 800-1000 μl extraction buffer into mortar and re-grind well.



3. Protocols

- Step 4.

- Transfer all the plant sap into 1.5 ml microcentrifuge tube and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

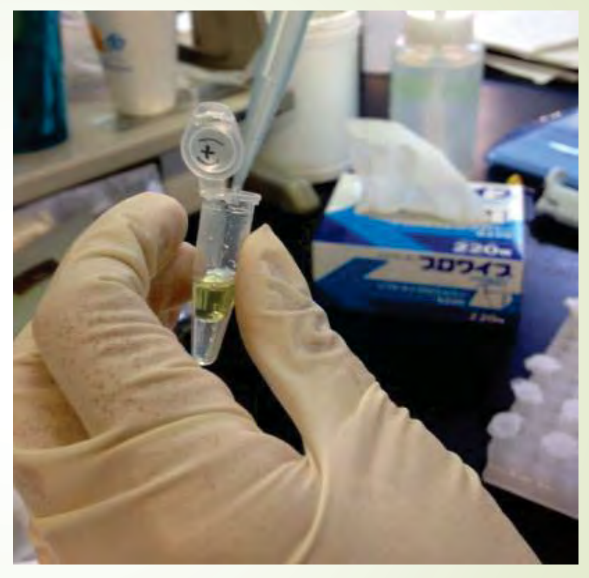
- After centrifuging, transfer 600 μl of supernatant into new 1.5 ml microcentrifuge tube.

3. Protocols

- Step 5.

- Add Phenol-Chloroform solution with equal volume of supernatant (600 μl).

- Vortex for few seconds and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.



3. Protocols

- Step 6.

- Repeat step 5 with 500 µl of supernatant and Phenol-Chloroform instead.

- Take 400 µl of supernatant and transfer it into new 1.5 ml microcentrifuge tube.

3. Protocols

- Step 7.

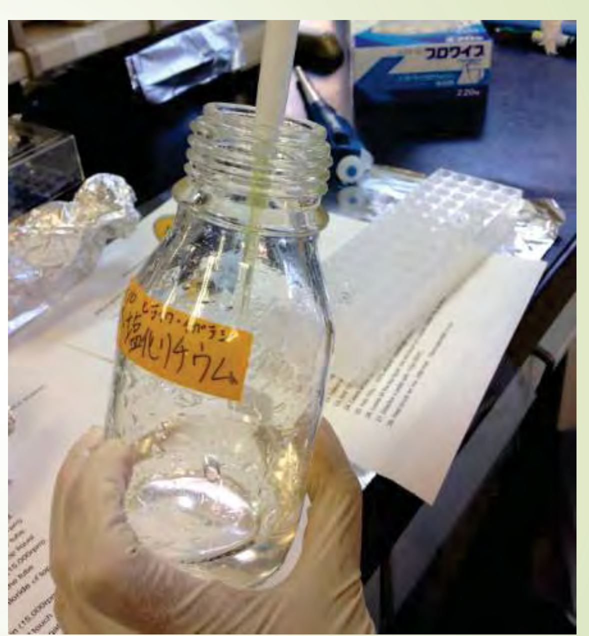
- Add 400 µl of Chloroform and centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

3. Protocols

- Step 8.

- Repeat step 7 with the volume of supernatant and chloroform added are 300 µl.

- Transfer 150 µl of supernatant into new 1.5 ml microcentrifuge tube and add 50 µl 8M lithium chloride (LiCl) (with 1:3 ratio (v/v)), vortex for few seconds and incubate in ice box for 1 hour.



3. Protocols

- Step 9.

- Centrifuge in cold condition (4°C) at 15,000 rpm for 20 minutes.
- Discard the supernatant and add 150 µl 70% ethanol.

3. Protocols

- Step 10.

- Centrifuge in cold condition (4°C) at 15,000 rpm for 5 minutes.

- Repeat step 14 and 15 with using 100% ethanol instead of 70% ethanol.

3. Protocols

- Step 11.

- Discard the supernatant and dry the pellet at room temperature for 10 minutes.

- Dissolve the pellet with 110 µl DEPC water.

4. Protocols

- Step 12.

- Put the tube with RNA in heat block at 65°C (Dry Thermo Unit) for 10 minutes, afterwards transfer to ice box for 10 minutes.

- Use the RNA for cDNA synthesis and PCR reaction, unless store RNA at -80°C.

Report 18. Method for purification of dsRNA of plant viruses from RNA-dissolving solution

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 14th, 2015

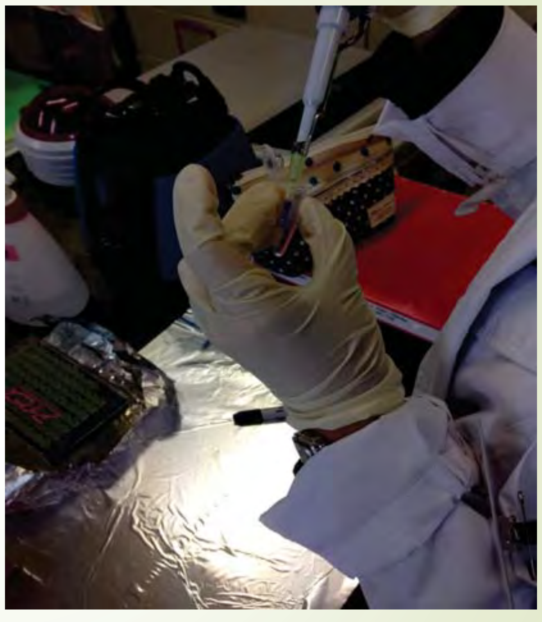
2. Samples and materials

- The dsRNA of *Cucumber mosaic virus* (CMV) was dissolved in RNA-dissolving solution (loading dye and RNase free water).

3. Protocols

- Step 1.

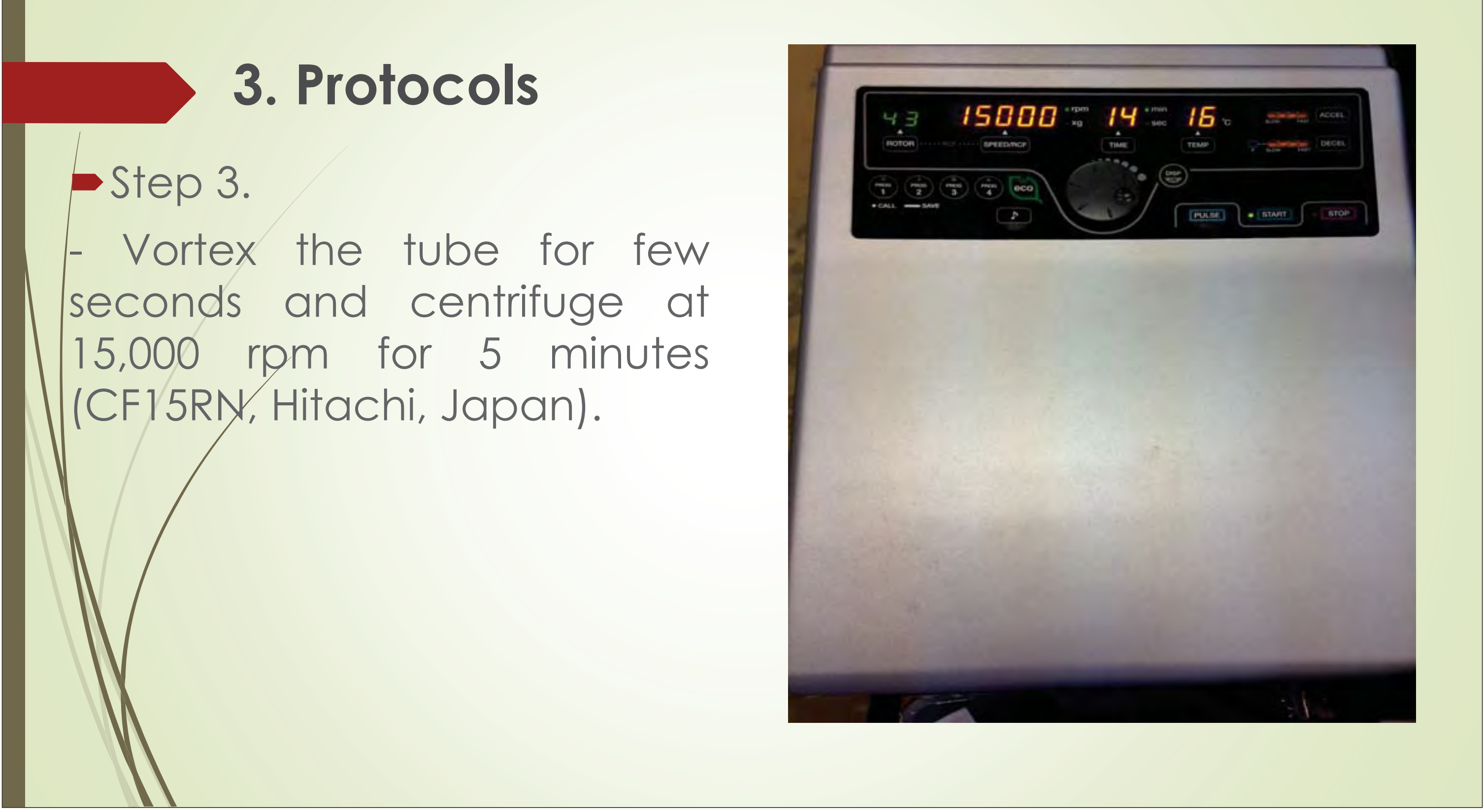
\- Transfer all the dsRNA solution into new 1.5 ml tube.



3. Protocols

- Step 2.

- Add 200 µl 1X TE buffer and 100 µl cold chloroform in 1.5 ml tube.



3. Protocols

- Step 4.

- Transfer 200 µl of supernatant to new 1.5 ml tube.

- Add cold 99.5% ethanol with twice amount of supernatant.

3. Protocols

- Step 5.

- Vortex for 1 minute and incubate at -80°C for 1 hour.

3. Protocols

- Step 6.

- Discard the supernatant using pipette and centrifuge at 15,000 rpm for 5 minutes.

- Discard the remaining supernatant and dissolve the dsRNA pellet in 20 µl 1X TE buffer.

- Keep the dsRNA at -80°C for the next using.

THANK YOU FOR YOUR ATTENTION

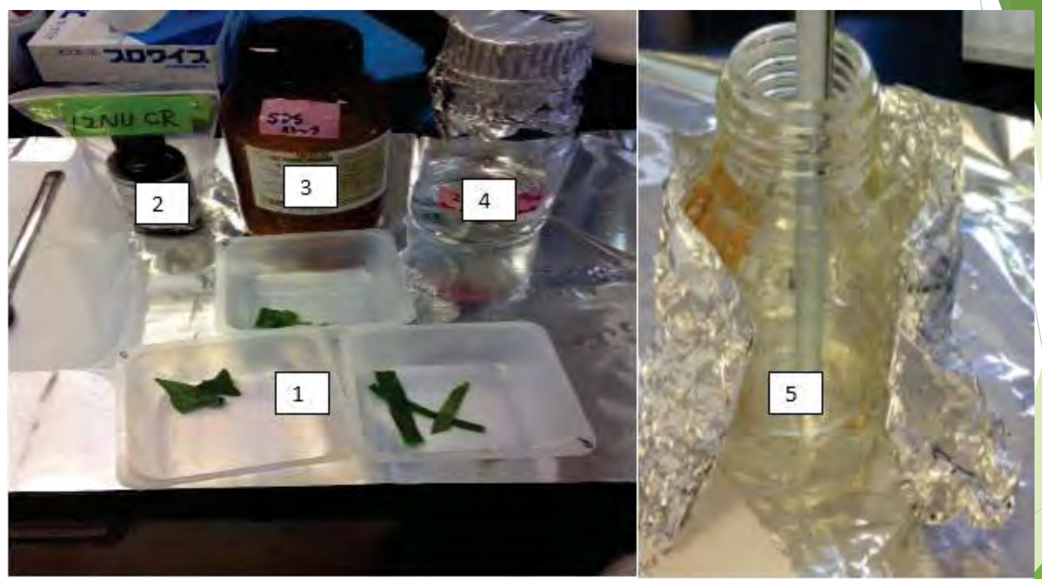
Viet Nam

Report 19. Detection of unknown-*potyvirus* on passionfruit plant

1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 7th-18th,2015

2. Materials and samples



1. Samples (potyvirus-inoculated passionfruit plant); 2. 2-mercaptoethanol; 3. SDS; 4. 2X STE buffer; 5. Phenol-Chloroform solution

3. Procedure

- RNA extraction
- cDNA synthesis, PCR assay and gel electrophoresis
- Gel extraction
- Ligation, transformation and cloning
- Miniprep, cycle sequence and DNA analysis

3.1. RNA extraction

- Conduct RNA extraction using phenol-chloroform method.
- The protocol of RNA extraction was described in previous report.

3.2. cDNA synthesis, PCR assay and gel electrophoresis

3.2.1. cDNA synthesis

- Prepare the cocktail mixture with required amount as follow:

5X RT buffer	4.0 µl
dNTP mixture (10 mM)	2.0 µl
Reverse primer of potyvirus universal primers	1.0 µl
RNAse inhibitor (10U/ µl)	1.0 µl
ReverTra Ace™	1.0 µl
Total RNA	11 µl
Total	20 µl

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.1. cDNA synthesis

- Carry out the cDNA synthesis with PCR conditions below:

Temperature (˚C)	Time (min.)
42	20
99	5
4	endless

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.2. PCR assay

- PCR assay was performed using *potyvirus* universal primer pairs. The expected band size will be 1.7 kbp.
- PCR conditions for *potyvirus* have been described as follow:

Temperature (˚C)	Time (min.)	Cycles
94	5	
94	0.5	34
47	1	
72	2	
72	10	

3.2. cDNA synthesis, PCR assay and gel electrophoresis (continued)

3.2.3. Gel electrophoresis

- Gel electrophoresis was conducted using 1.5% agarose gel.
- The protocol of gel electrophoresis performance was presented in previous report (see report 7)

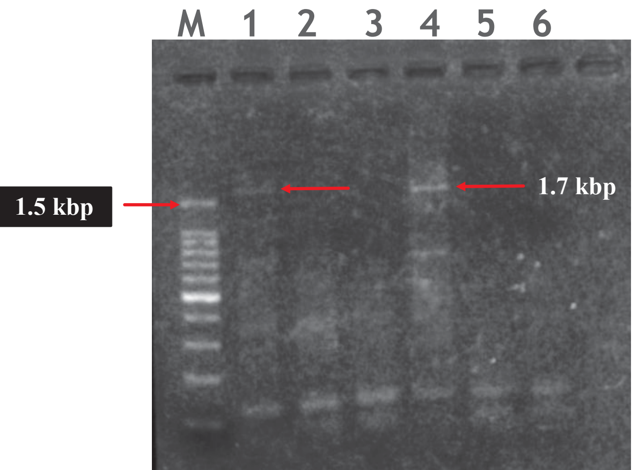


Fig 1. PCR assay of unknown *Potyvirus* inoculated passionfruit plant collected from HOGOKEN Lab. with *potyvirus* universal primers. The PCR band with the size of ~1.7 kbp (red arrows) was amplified from positive control (lane 1 & 4). No band was amplified from *Potyvirus* inoculated plant (lanes 2 & 5) and negative control (lanes 3 & 3). The 100bp DNA ladder (Promega, USA) was included as marker.

3.3. Gel extraction

- Gel extraction was performed using Wizard SV Gel and PCR clean-Up system (Promega, USA).
- Procedure of gel extraction was presented in previous report (see report 13)

The Wizard SV Gel and PCR clean-Up system (Promega, USA)
1. Membrane Binding Solution; 2. Membrane Wash Solution; 3. Nuclease-Free Water; 4. Minicolumn; 5. Collection Tube

3.4. Ligation, transformation and cloning

3.4.1. Ligation

- Ligation was carried out using LigaFast™ Rapid DNA Ligation System (Promega, USA).
- Procedure of ligation was presented in previous report (see report 13)

The LigaFast™ Rapid DNA Ligation System (Promega, USA)
1. pGEM-T vector; 2. 2X rapid ligation buffer; 3. T4 DNA ligase

3.4. Ligation, transformation and cloning (continued)

3.4.2. Transformation

- The purpose of this step is to transfer the unknown *potyvirus* inserted pGEM vector into *E. coli* plasmid.
- The procedure of transformation was described in previous report (see report 14).

3.4. Ligation, transformation and cloning (continued)

3.4.3. Cloning of recombinant plasmid

- Cloning of recombinant plasmid was conducted on TB medium
- The procedure of cloning was described in previous report (see report 14).

Annex 20

Name: **Tran Van Chien**

Country: **Vietnam**

REPORT ON VISIT STUDY TO YOKOHAMA PLANT PROTECTION STATION

1. Place and time

Place: Yokohama plant protection station.

Time: Dec. 11th, 2015

2. Activities in Yokohama plant protection station

2.1. Having a presentation on Plant Quarantine System in Japan

Japan's Plant Quarantine is an organization under the Ministry of Agricultural, Forestry and Fisheries (MAFF). In line with global market, the varieties and quantities of crops to be imported to Japan will increase and causing greater risk of plant pests and diseases introduction. The objective of quarantine is to prevent the introduction and spread of pests in all areas of Japan.

Fig. 1: Presentation on Plant Quarantine System in Japan

Plant protection have function to protect Japan's agriculture resources. Japan plant quarantine stations implement quarantine procedures that target both domestic and overseas products. Such quarantine procedure include import quarantine to prevent the introduction of overseas plant pests, export quarantine in response request from other countries and domestic quarantine to control pests in Japan.

Japan's plant quarantine system consist of:

1. International Plant Quarantine
   a. Import Quarantine (Import plant inspection, post entry quarantine, and pre-shipment quarantine)
   b. Export Quarantine (export plant inspection and field inspection of export plant)
2. Domestic Plant Quarantine
   a. Quarantine of Domestic Seed and Seedlings
   b. Eradication/control Program for Designated Pests
   c. Monitoring Survey for Newly Invasive Pests
   d. Emergency Action

The major plant pests and diseases requiring precaution for Japan are Mediterranean Fruitfly (*Ceratitis capitata*), Fire Blight disease (*Erwinia amylovora*), Coddling Moth (*Cydia pomonella*), Tobacco Blue Mold (*Peronospora tabacina*), and others.

2.2. Visit the Yokohama plant protection station's exhibition

After the presentation, we had an opportunity to see the Yokohama plant quarantine station's exhibition to more understand about the foundation, history and development of Plant Quarantine System in Japan as well as Yokohama plant protection station.

Fig. 2: Visiting the Yokohama plant protection station's exhibition

2.3. Visiting the plant quarantine facilities of Research Center, Yokohama plant protection station.

Beside Plant Quarantine Station, we also visited Center Research where support the system of plant quarantine in technical aspect. Center Research have some Section such as:

1. Disinfestation Technology section to develop new technology about quarantine treatment
2. Entomology and Nematology Section: a laboratory that had work on developing new method about insect and nematode identification
3. Plant Pathology Section: a laboratory that had work on developing new method about identification of plant pathogen
4. Pest Risk Assessment Section: Work on assessment of quarantine pest
   Pest risk analysis (PRA) evaluates scientific evidence to determine whether an organism is a pest. If so, the analysis evaluates the probability of introduction and spread of the pest and the magnitude of potential economic consequences in a defined area, using biological or other scientific and economic evidence.
5. Pest Risk Management Section: Work on risk management of quarantine pest including recommendation of quarantine treatment based on result of pest risk assessment. If the risk is deemed unacceptable, the analysis may continue by suggesting management options that can reduce the risk to an acceptable level. Subsequently, pest risk management options may be used to establish phytosanitary regulations.
6. Living Modified Organism Section: Work on living modified organism research based on Cartagena Protocol and Biosafety to the Convention on Biological Diversity. This section seeks to protect biological diversity from

the potential risks posed by genetically modified organisms resulting from modern biotechnology

7. Pest Identification Section: work on identify quarantine pest including plant pest and pathogen

Fig. 3: Visiting the plant quarantine facilities of Research Center, Yokohama plant protection station

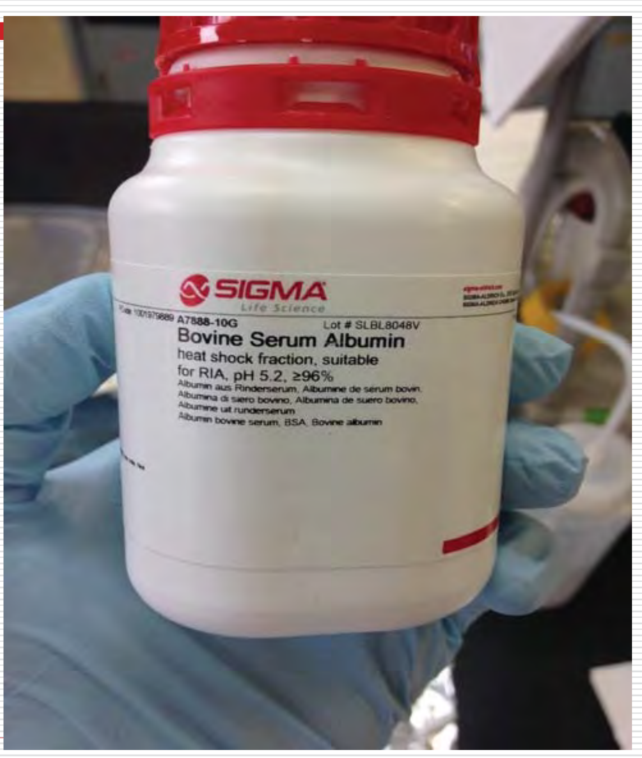
Report 21. Method for SDS PAGE (Sodium Dodecyl Sulfate PolyAcrylamide) electrophoresis

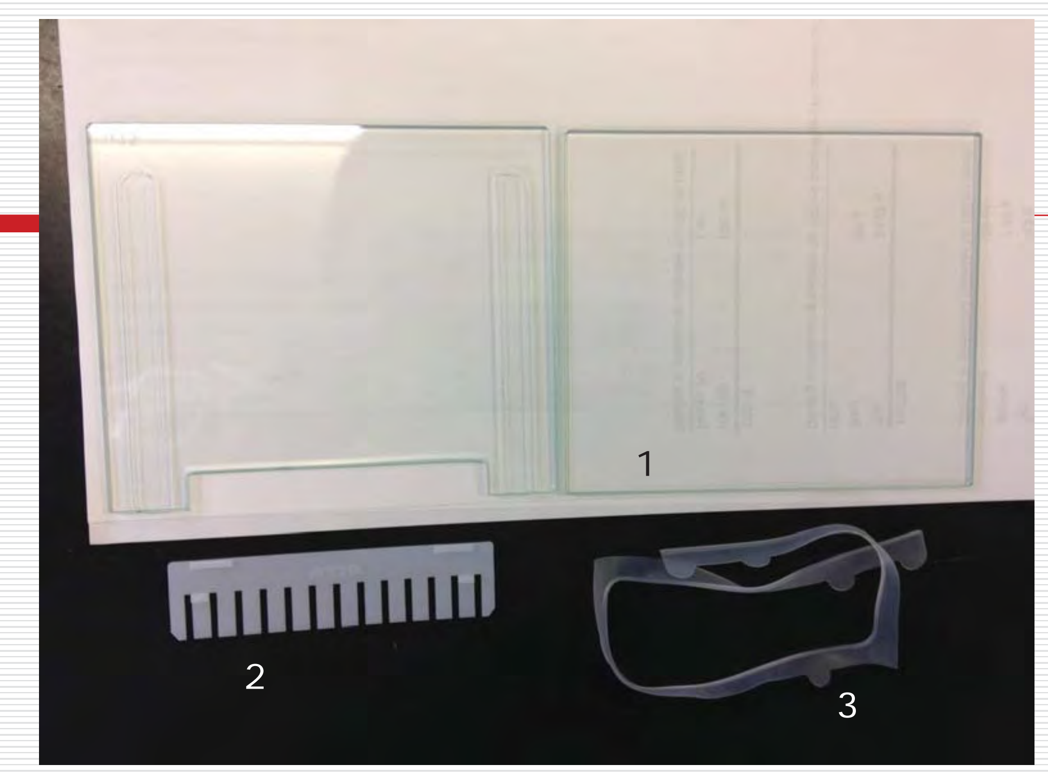
1. Place and time

- Place: HOGOKEN Lab., Department of International Agricultural Development, Tokyo University of Agriculture (Tokyo NODAI).
- Time: Dec. 15th, 2015

2. Samples and materials

- The protein used to conduct the experiment was Bovine Serum Albumin (BSA) (Sigma, USA).





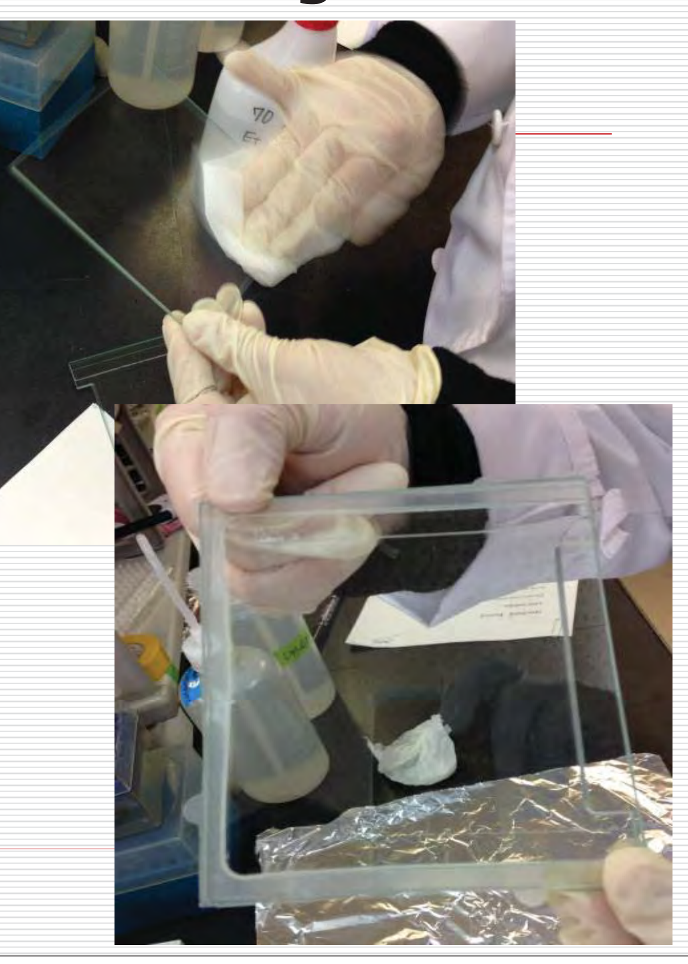
1: Glass plates; 2: Comb; 3: Spacer

3. Protocols

3.1. Preparation of SDS PAGE gel

- Step 1.

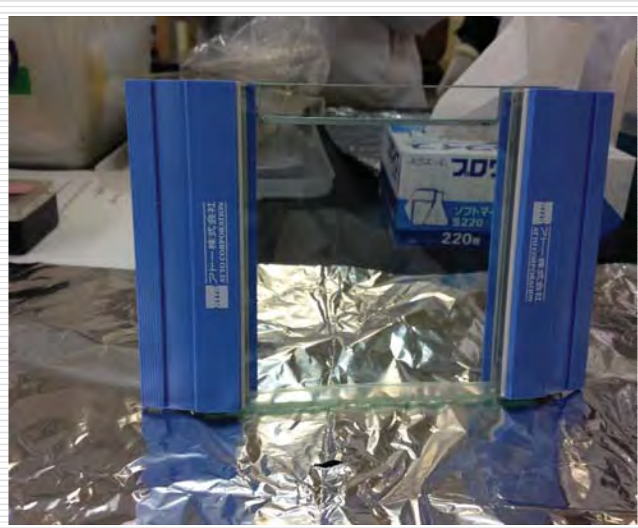
- Wipe glass plates, spacer and comb by 70% ethanol.
- Assemble the glass plates with spacers.



3.1. Preparation of SDS PAGE gel

- Step 2.

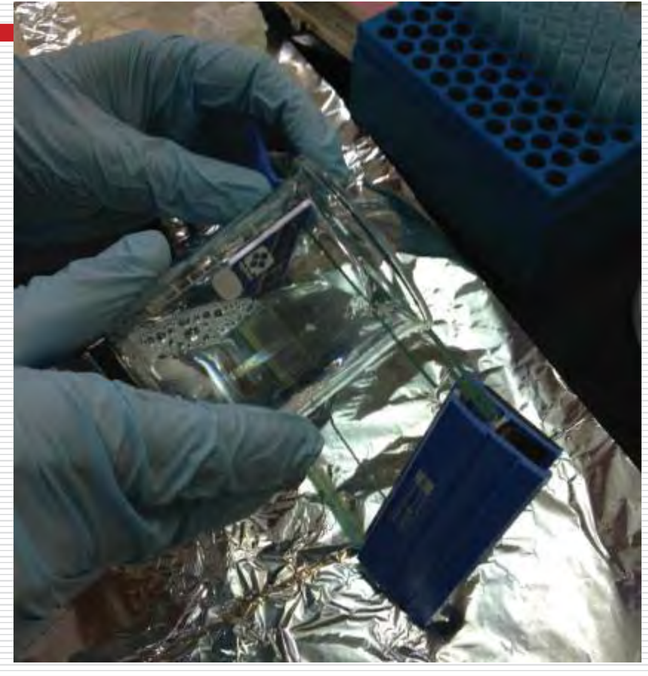
  - Secure gel glass assembly with bull clips on each side.



3.1. Preparation of SDS PAGE gel

- Step 3.

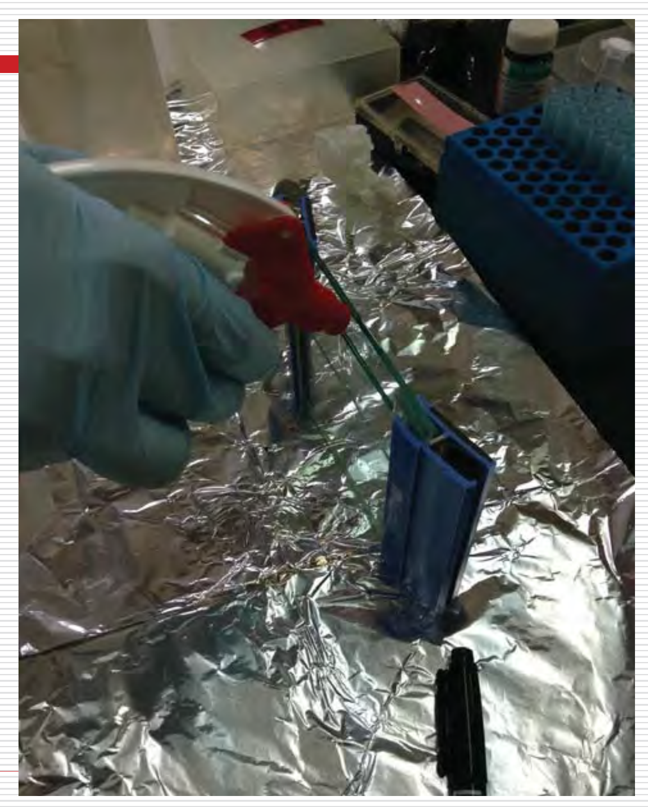
  - Pour the separating gel into space of gel plate assembly up to 2/3 of height of gel plate assembly.



3.1. Preparation of SDS PAGE gel

- Step 4.

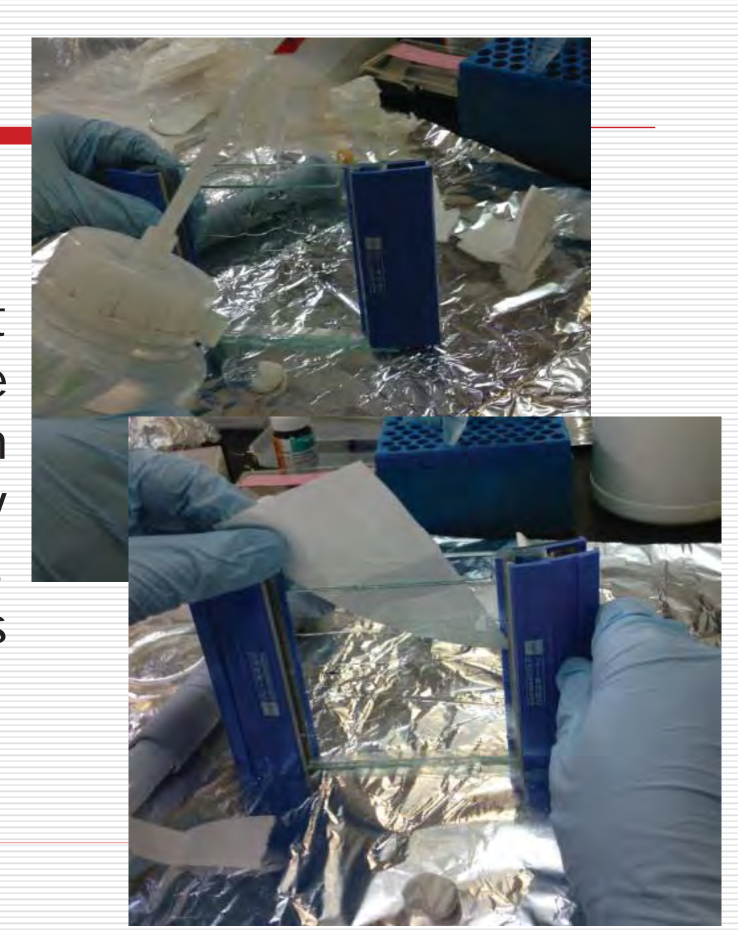
- Pour little amount of 70% ethanol into the space between two glasses to eliminate totally bubbles .



3.1. Preparation of SDS PAGE gel

- Step 5.

- After the gel get solidification, remove the ethanol and wash the surface of gel by water and filter paper. Do not scratch gel's surface.

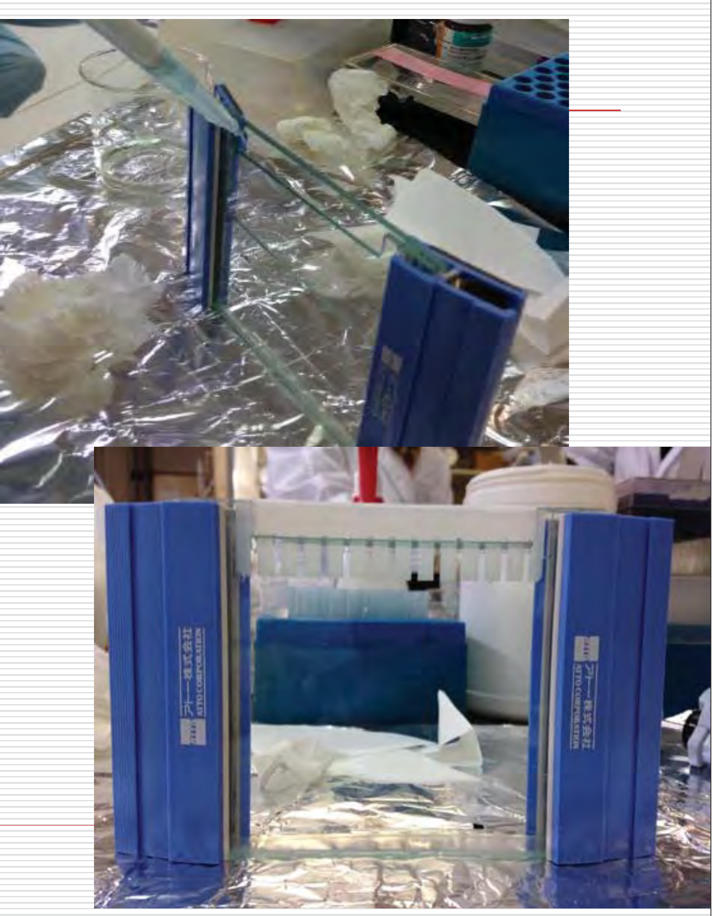


3.1. Preparation of SDS PAGE gel

- Step 6.

- After removing the ethanol, use pipette to pour the staking gel onto the separating gel. Do not make bubbles.

- Finally, insert the comb into the space.

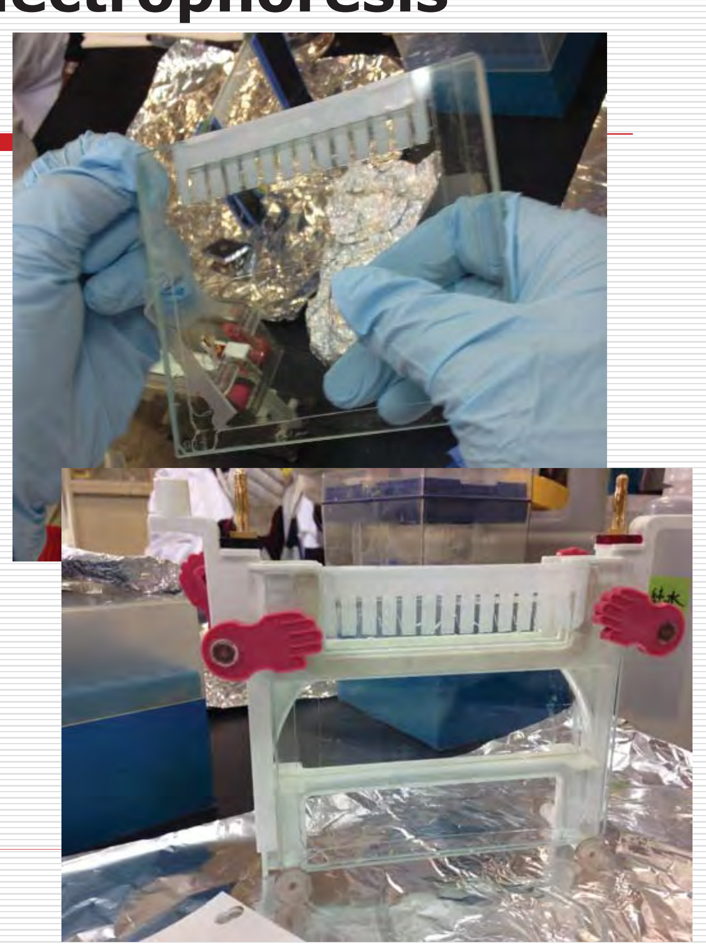


3.2. SDS PAGE gel electrophoresis

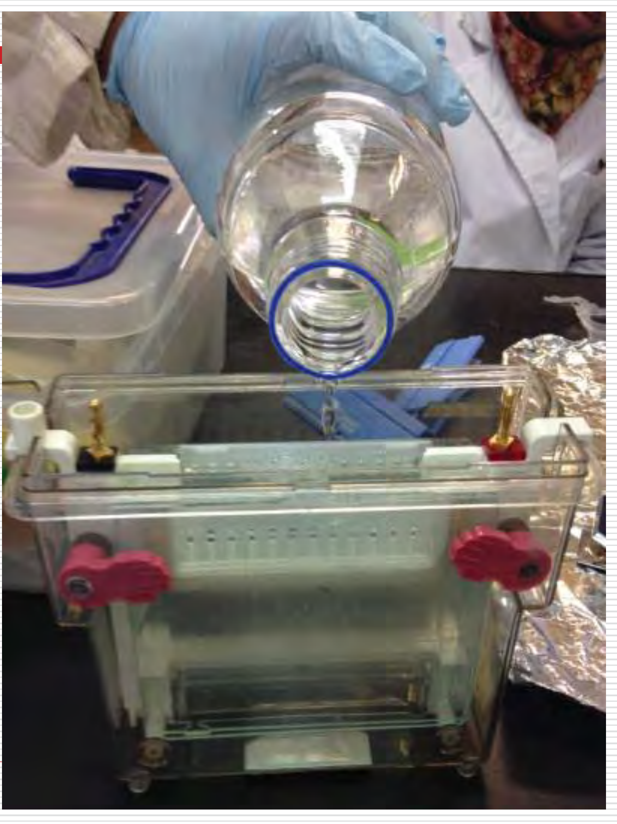
- Step 1.

- Remove the spacer from gel plate assembly.

- Put gel plate assembly into electrophoresis tank.



3.2. SDS PAGE gel electrophoresis



- Step 2.

- Pour 1X electrophoresis buffer into electrophoresis tank. The top of the gel is completely submerged in the buffer.

- For two side spaces of gel plates, pour the buffer up to ½ of the height of gel plates.

- Remove the bubbles inside the tank using syringe.

3.2. SDS PAGE gel electrophoresis

- Step 3.

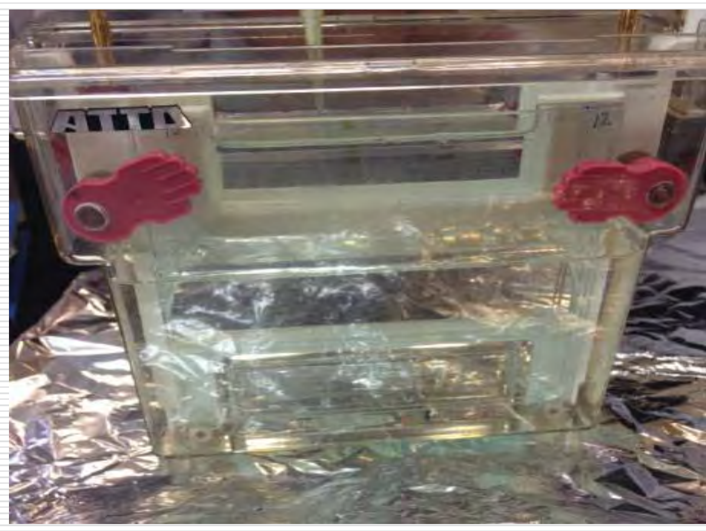
- Carefully remove the comb from the gel plates. Adjust the shape of wells using syringe.

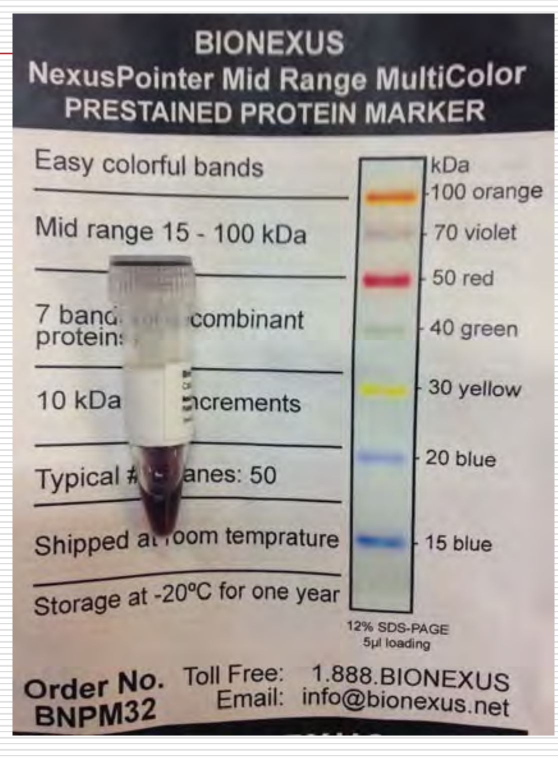
- Mix the same volume of the sample and sample buffer. Afterward, heat the mixture at 100°C for 5 minutes.

3.2. SDS PAGE gel electrophoresis

- Step 4.

- Load the marker (Bionexus, USA) and heated samples into the wells.



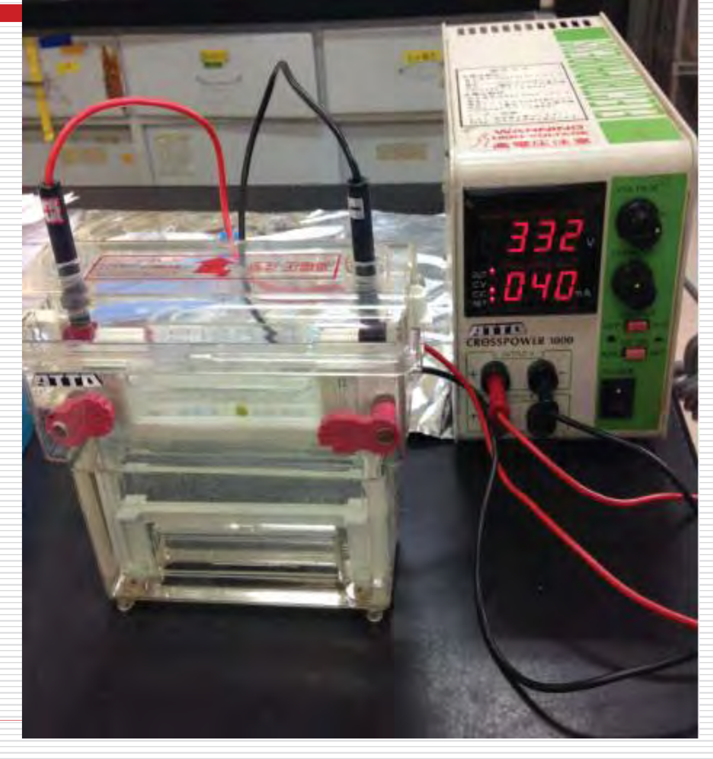


3.2. SDS PAGE gel electrophoresis

- Step 5.

- Run the electrophoresis initially at (20 x number of gel plate) mA. After the protein bands pass through the stacking gel, increase the electricity to (40 x number of gel plate) mA.

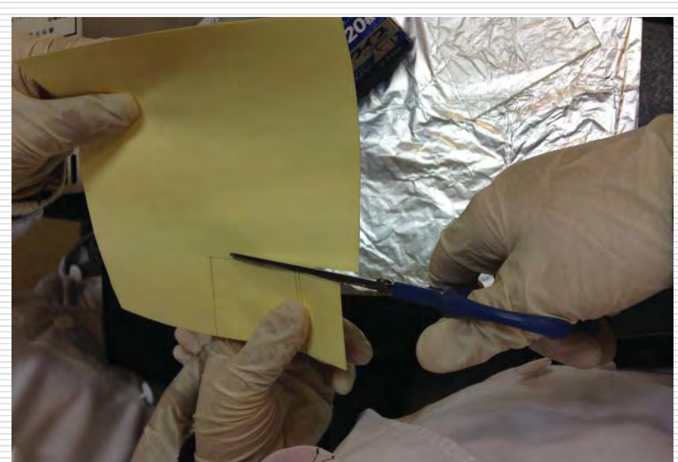
- The electrophoresis will be finished when dye front is near the bottom of the gel (above 5 mm).



3.3. Protein transfer

- Step 1.

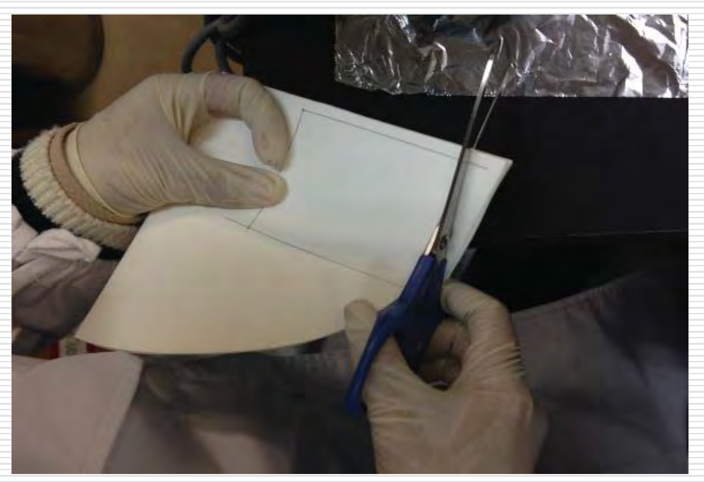
- Cut the Nitrocellulose Membranes (BIO-RAD, Germany) to fit with the size of gel.



3.3. Protein transfer

- Step 2.

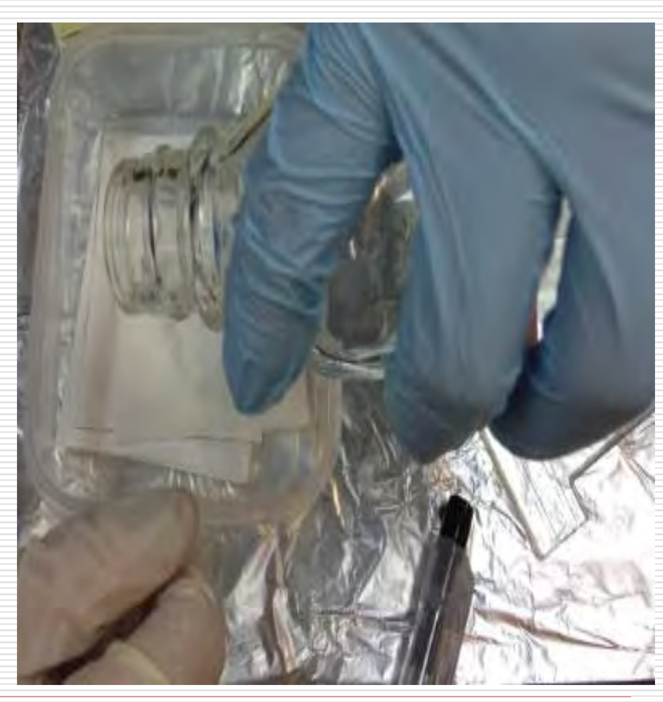
- Prepare the filter papers having the length and width are 1 cm larger than membrane.



3.3. Protein transfer

- Step 3.

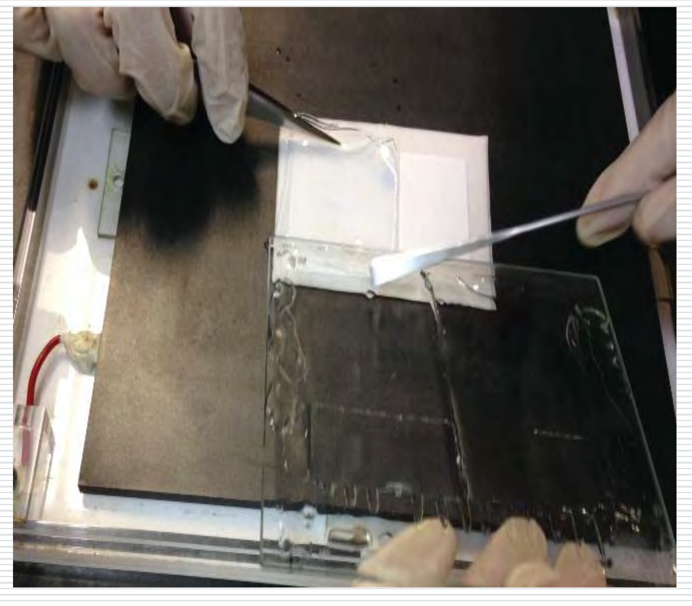
- Make the filter paper wet by soaking in transfer buffer.



3.3. Protein transfer

- Step 4.

- Put the membrane, gel and filter papers into the transfer device in order: three filter papers at the bottom, gel, membrane and three filter papers at the top.

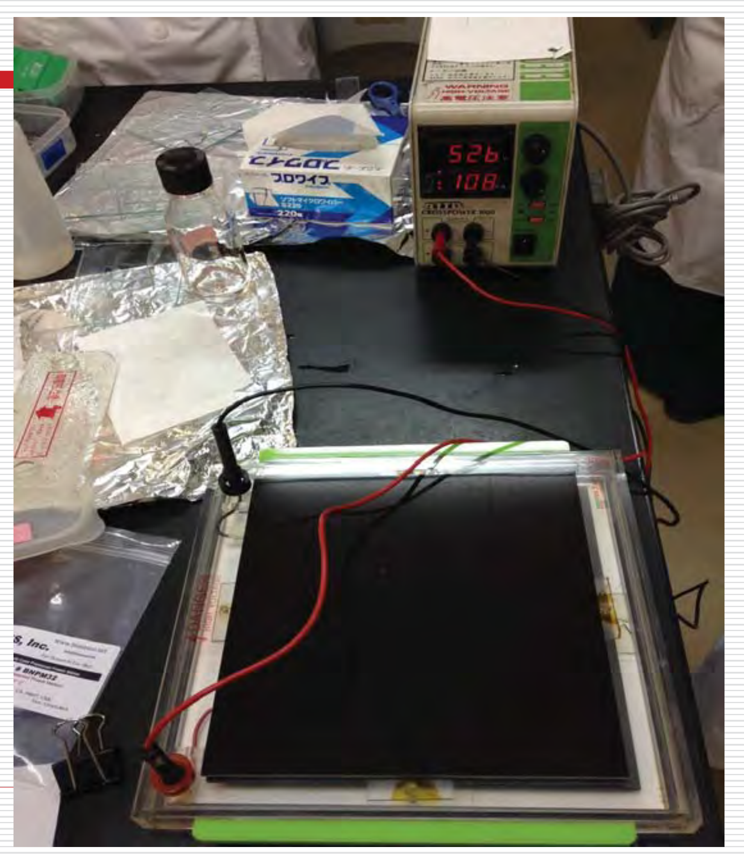


3.3. Protein transfer

- Step 5.

- Conduct the transferring process by using the electricity at 40V, 20W, (2 x membrane area) mA for 1 hour.

- For example: if the area of membrane is 20 cm^2, the mA of electricity will be set at (2 x 20) = 40 mA.



3.4. Detection of protein on membrane

- Step 1.

- After 1 hour, soak membrane in staining solution for protein gel (Instant Blue, Expedeon, UK) for 2 minutes.

3.4. Detection of protein on membrane

- Step 2.

- Shake the membrane with staining solution for 5 minutes using shaker.

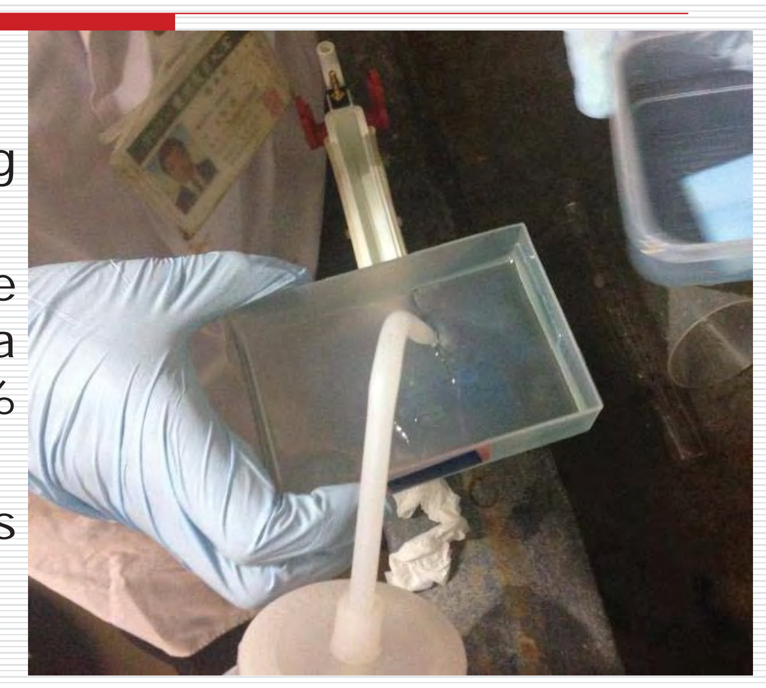
3.4. Detection of protein on membrane

- Step 3.

- Pour off the staining solution.

- Submerge membrane in distilled water added a small amount of 70% ethanol.

- Shake for 3 minutes using shaker.

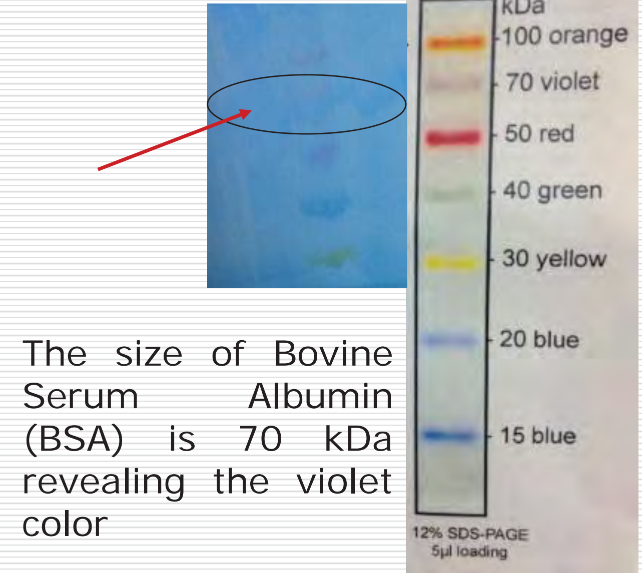


3.4. Detection of protein on membrane

- Step 4.

- Pour off solution.

- Observe the protein band on the membrane by naked eyes.



The size of Bovine Serum Albumin (BSA) is 70 kDa revealing the violet color

THANK YOU FOR YOUR ATTENTION

Attachment 5

Training Workshop on Diagnostics of Leafminers of Agricultural Importance

29 February - 11 March, 2016

Research Center for Biology, Indonesian Institute of Science (LIPI), Cibinong, Bogor, Indonesia

REPORT

Organized by:

Museum Zoologicum Bogoriense, LIPI

In Collaboration with:

ASEAN Network on Taxonomy

2016

REPORT

TRAINING WORKSHOP ON DIAGNOSTICS OF LEAFMINERS OF AGRICULTURE IMPORTANCE

Bogor, 29 February -11 March 2016

<u>Dr. Hari Sutrisno</u>
Regional Training Coordinator

Division of Zoology
Research Center for Biology
The Indonesian Institute of Science
2016

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Compilation of Lecture Notes

1. **Dr. Hirosaki Sato**
2. **Dr. Mallik Malipatil**
3. **Dr. Hari Sutrisno**
4. **Prof. Rosichon Ubaidillah**

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Acknowledgments

Training Workshop on the Diagnostic of leafminers of agriculture importance that was implemented by Museum Zoologicum Bogoriense, The Indonesian Institute of Science through the ASEAN Plant Health Cooperation Network (APHCN) of ASEANET project on "Taxonomic capacity building to support market access for agricultural trade in the ASEAN region" has been succesfully conducted from 29 February to 11 March 2016. This training workshop has provided participants with basic and practical understanding of the concept of leafminers, identification of important pest leafminers, and management practices for them.

We would like to thanks Dr. Lum Keng Yeang – Chairperson, APHCN – ASEANET and Dr. Soetikno S. Sastroutomo – Secretary, APHCN – ASEANET for the collaboration to conduct this training workshop. Our gratitude thanks also go to all resources persons, Dr. MB. Malipatil from La Trobe University, Dr. Sato Hiroaki from Nara Women University, Dr. Rosichon & Mr. Zein MSc from The Research Center for Biology, who patiently trained the participants and provided paticipants with a lot of information on the leafminer and improved their skill in identification of leafminer both based on morphological and molecular characters. Our thanks also go to all team members of regional coordinator and technical assistants who supported all the training fasilities from preparing the training material and arranging the laboratorium/class and assisted the trainers during fieldwork.

Our thanks also go to the Japan – ASEAN Integration Fund (JAIF) which financially supported this training, without this grant it is imposisible to conduct this training succesfully.

Bogor, Maret 2016

Regional Training Coordinator

EXECUTIVE SUMMARY

Training Workshop on the Diagnostic of leafminer of agriculture importance was implemented by Museum Zoologicum Bogoriense-The Indonesian Institute of Science through the ASEAN Plant Health Cooperation Network (APHCN) of ASEANET project on **"Taxonomic capacity building to support market access for agricultural trade in the ASEAN region"**. The project is funded by the Japan – ASEAN Integration Fund (JAIF) that will be implemented for two years covering several activities related to training and attachment programs.

This two-week training workshop (February 29 –March 11, 2016) was participated by 20 participants from 10 Asean Countries (Brunei Darussalam, Cambodia, Indonesia, Lao PDR, Malaysia, Myanmar, Philippines, Thailand, Vietnam and Singapore). Majority of them are staff of the Plant Quarantine Centre and Plant Protection Division under the Department of Agriculture.

The main goal of the training workshop was to develop capacity building among entomologists across the ASEAN region in addressing leafminer pests existing in each country that may pose potential threats (emerging or invasive) in the exchange of crops or planting materials. The training workshop provided the participants with basic knowledge on ecology and taxonomy of leafminer of agriculture importance with focused on improvement of identification skill of leaf miner and their parasitoid by using morphological characters and molecular technique.

The training course utilized the combination of theory, laboratory practical, and field trip. Theory class and identification was conducted in the seminar Room of Museum Zoologicum Bogor. This room has been modified into an identification room completed with 20 stereo microscope, 10 compound microscope and internet access. Laboratory practical for identification of leafminer by using molecular technique was conducted in the Genetic laboratory of Museum Zoologicum Bogoriense.

Pre-test and Post-test were conducted to evaluate the improvement of the knowledge and the skill of the participants and also to select the candidate to follow the 3 months-attachment program in Japan under supervision Dr. Sato, in Australia under supervision Dr. Malipatil, and in Indonesia under supervision Dr. Rosichon & Dr. Hari. The non-technical post-test also was conducted to evaluate the organizing of the training program.

1. Project Out Line

1.1. Title of the project: Workshop Training on Diagnostics of Leafminer of Agriculture importnace

1.2. Background

Leafminers are an insect group whose larvae feed on leaf tissues within leaves during a part or the whole of their larval stage, including Coleoptera (beetles), Diptera (flies), Hymenoptera (sawflies) and Lepidoptera (moths). Leafminers of agriculture importance are found mainly in Diptera and Lepidoptera. Of them, dipteran leafminers of the genera *Liriomyza*, *Phytomyza* and *Agromyza* (Agromyzidae) are the most serious pests of crops, ornamentals and vegetables throughout the world. Lepidopteran leafminers often cause serious damage to fruit trees. *Phyllocnistis citrae* (Gracillariidae) mines leaves of citrus plants and inflict a heavy damage on the plants. Apple blotch leafminers (*Phyllonorycter* spp., Gracillariidae), the horse chestnut leafminer (*Cameraria ohridella*, Gracillariidae) and the tea leaf-roller (*Caloptilia theivora*, Gracillariidae; larvae of this species mine leaves during the first three instar stages) are famous as lepidopteran pest leafminers.

Damage by some leafminers on horticultural plants is conspicuous also in South-east Asia. Particularly, several species of *Liriomyza* have invaded since 1990' and become major pests of vegetable crops such as tomatoes, cucumbers, egg plants, lettuce, spinach and legumes. Accordingly, the management of these pests is a central issue in the field of horticulture and applied entomology. However, it is difficult to identify the species of *Liriomyza* leafminers, because they are polyphagous and their mines are very similar to each other, to say nothing of larvae. This can delay an effective management of the concerned leafminer. Similar problems could occur in other leafminers.

In this workshop, we aim to teach the participants how important it is to study taxonomy and ecology of leafminers from the viewpoint of pest management and instruct them in taxonomic skills in identifying the species of leafminers by morphological characters and DNA barcoding.

1.3. Course Description

This workshop is coordinated by Research Center for Biology, Indonesian Institute of Science through the ASEAN Plant Health Cooperation Network (APHCN) of ASEANET project on "Taxonomic capacity building to support market access for agricultural trade in the ASEAN

region." This project is funded by the Japan-ASEAN Integration Fund that will be implemented for 2015-16 covering several activities related to training and attachment programs.

This workshop aims to provide participants with basic and practical understanding of the concept of leafminers, identification of important pest leafminers, and management practices for them. To do this, the training course covers the following topics: basic taxonomy and ecology of major species, genera and families of pest leafminers, collecting and preparation of specimens, identification of mines, immatures and adults based on morphological characters and DNA barcoding, natural enemies, especially, parasitoids, impacts of leafminers on major agricultural crops in South-east Asia, and available options of pest leafminer management in reducing the loss of crop yield. Thereby, participants will be able to design effective plans for controlling pest leafminers.

1.4. General Objectives

1.4.1. Lecture

At the end of the training, the participants will acquire knowledge about the concept of leafminers, how seriously they damage horticultural crops, and how to control them appropriately.

1.4.2. Laboratroy

At the end of the training, the participants will acquire taxonomic skills in identifying the species, genera and families of major pest leafminers on the basis of external characters and DNA barcoding, collecting, preparing and labeling specimens, and making slide preparations of genitalia.

1.5. Specific Objectives

1.5.1. Lectures:

- To acquire taxonomic and ecological knowledge of pest leafminers and their related species.
- To be aware of how seriously leafminers damage crops.
- To be aware that major pest leafminers have been invaded from other regions.
- To learn how to protect crops from leafminers through integrated pest management.
- To gain knowledge of parasitoids attacking pest leafminers and their application to biological control.

1.5.2. Laboratory:

- To detect crop leaves mined by a diversity of leafmining larvae.
- To learn basic techniques for collecting in the field and rearing leafminers in the laboratory.
- To learn how to make slide preparations of genitalia.
- To identify the taxon (species, genus, family and order) of leafminers on the basis of morphology and DNA bar-coding.
- To learn DNA bar-coding and its application.

1.6. Training Methodology

The training will be conducted by a combination of interactive lectures, laboratory practices and field visits. Field observation and sampling will be carried out not only in crop fields but also in semi-natural fields.

1.7. Training Contents

The following is the Training Program:

DATE/VENUE/TIME	TOPIC/ ACTIVITY	RESOURCE PERSON(S) /FACILITATOR
Sunday, 28 February 2016		
	Arrival and billeting at AMARIS Pakuan Hotel, Bogor	Dr.Soetikno S.S., ASEANET
	WEEK I	
Monday, 29 February 2016		
Venue: Seminar Room	SESSION 1. OPENING AND INTRODUCTION	
08:45 - 9:00	Registration	Ms. Gina RCB-LIPI
09:00 - 9:30	Welcome address	Dr. Hari Sutrisno RCB-LIPI
	Opening Remark 1	Dr. Lum Keng Yeang Project Manager &

		Chairperson ASEANET
	Opening Remark 2	Dr.Witjaksono Head of RCB-LIPI
09:30 - 9:40	Workshop context and overview	Dr.Hari Sutrisno RCB-LIPI
09:40 - 10:00	Introduction of participants, trainers and training team	Ms.Vany RCB-LIPI
10:00 - 10:15	Group photograph	Mr Arid RCB-LIPI
10:15 - 10:30	Tea/coffee break	
10:30-10:45	Pre Test	Dr. Hari Sutrisno RCB-LIPI
Venue: Seminar Room	SESSION 2. LEAFMINER BIOLOGY INTRODUCTION	
10:45 - 12:00	Lecture: Biology and ecology of leafminers	Dr. Hiroaki Sato Nara Women's University
12:00 - 13:00	Lunch break	
13:00 - 14:30	Lecture: Economic importance of leafminers	Dr. M.B. Malipatil La Trobe University
14:30 - 15:00	Lecture: Basic classification of leafminers—Diptera	Dr. M.B. Malipatil La Trobe University
15:00 - 15:15	Tea/coffee break	
15:15 - 16:00	SESSION 3. DIPTERAN LEAFMINER RESOURCES ON THE WEB Lecture and practice: Dampewolf website, Joshi's website, Parella's biology of leafminers, and EU diagnostic protocols	Dr. M.B. Malipatil La Trobe University
16:00 - 17:00 17:00- 18:00	SESSION 4. CABI CROP PROTECTION COMPENDIUM Lecture and demonstration: Use of CABI CPC for data on pest biology, distribution, images and risk analysis	Dr. M. B. Malipatil La Trobe University

	Reserve	
18:00 - 20:30	DINNER RECEPTION Front Hall Widyasatwaloka Building	All participants & Resource Persons
Tuesday,1 March 2016		
7:00-17:00	FIELD TRIP to Agatho Farm Cisarua-West Java	Dr. Hari Sutrisno + 4 Entomology staff, Dr. Hiroaki Sato & All participants Resource Persons
Wednesday, 2 March 2016		
Venue: Seminar Room	SESSION 5. PREPARATION OF DIPTERAN LEAFMINER SPECIMENS COLLECTED IN THE FIELD	
08:30 - 10:00	Practice: Demonstration	Dr. M.B. Malipatil La Trobe University
10:00 - 10:30	Tea/coffee break	
	SESSION 6. DISSECTION AND SLIDE PREPARATION OF DIPTERAN LEAFMINER GENITALIA	
10:30 - 12:00	Practice: Demonstration	Dr. M.B. Malipatil La Trobe University
12:00 - 13:00	Lunch break	
13:00 - 15:30	Hands-on work	Dr. M.B. Malipatil La Trobe University
15:00 - 15:30	Tea/coffee break	
15:30-17:00	Hands-on work	Dr. M.B. Malipatil La Trobe University
Thursday, 3 March 2016		
Venue: Seminar Room	SESSION 7. IDENTIFICATION OF DIPTERAN LEAFMINERS	
08:30 - 10:00	Lecture: Morphology and terminology	Dr. M.B. Malipatil La Trobe University

10:00 - 10:30	Tea/coffee break	
10:30 - 12:00	Practice: Identification of specimens using CSIRO fly CD and paper keys	Dr. M.B. Malipatil La Trobe University
12:00 - 13:00	Lunch break	
13:00 - 14:30	Practice: Identification of specimens using keys	Dr. M.B. Malipatil La Trobe University
15:00 - 15:30	Tea/coffee break	
15:30 - 17:00	Practice: Identification of specimens using keys	Dr. M.B. Malipatil La Trobe University
Friday, 4 March 2016		
Venue: Seminar Room	SESSION 8. LEAFMINER BIOLOGY INTRODUCTION: Diptera	
8.30-.9:00	Review	Dr. M. B. Malipatil La Trobe University
	SESSION 9. PARASITOIDS ASSOCIATED WITH Dipteran LEAFMINERS	
9.00-10:00	Lecture: Biology of parasitoids associated with Diptera leafminers	Prof. Dr. Rosichon RCB-LIPI
10:00-10:15	Tea Break	
10:15-11:30	Lecture: Biology of parasitoids associated with Diptera leafminers	Prof. Dr. Rosichon RCB-LIPI
11:30-13.30	Lunch break	
11:30-16.30	Identification of leafminer	Prof. Dr. Rosichon RCB-LIPI
Saturday, 5 March 2016		
Venue: Hotel 7.00-17.00	FIELD TRIP Fruit Garden Mekarsari-Cileungsi	Dr. Hari Sutrisno + 4 Entomology staff, Dr. Hiroaki Sato & All participants Resource Persons
Sunday, 6 March 2016		
	REST DAY	

	WEEK II	
Monday, 7 March 2016		
Venue: Seminar room	SESSION 10. DISSECTION AND PREPARATION OF LEPIDOPTERAN LEAFMINER GENITALIA	
08:30 - 10:00	Lecture: Rearing methods	Dr. Hiroaki Sato Nara Women's University
	Lecture: Morphology and terminology	Dr. Hiroaki Sato Nara Women's University
10:30 - 12:00	Practice: Demonstration	Dr. Hiroaki Sato Nara Women's University
12:00 - 13:00	Lunch break	
13:00 - 14:30	Practice: Hands-on work	Dr. Hiroaki Sato Nara Women's University
15:00 - 15:30	Tea/coffee break	
15:30 - 17:00	Practice: Hands-on work	Dr. Hiroaki Sato Nara Women's University
Tuesday, 8 March 2016		
Venue: Seminar room	SESSION 11. PREPARATION OF WINGS AND LARVAE	
08:30 - 10:00	Practice: Demonstration	Dr. Hiroaki Sato Nara Women's University
10:00 - 10:30	Tea/coffee break	
10.30 – 12.00	Practice: Hands-on work	Dr. Hiroaki Sato Nara Women's University
12:00 - 13:00	Lunch break	

13:00 – 15.00	SESSION 12. IDENTIFICATION OF LEPIDOPTERAN LEAFMINERS	
	Practice: Identification of specimens using keys	Dr. Hiroaki Sato Nara Women's University
15.00 - 15.30	Tea/coffee break	
15.30 - 17.00	SESSION 13. Larva & Genitalia Slide of Lepidoptera	Dr. Hiroaki Sato Nara Women's University
Wednesday, 9 March 2015		
Venue : Seminar room	SESSION 14. DNA BARCODING DNA Barcoding	
08.30 -10.00	Lecture: Extraction of DNA from leafminers	Dr.HariSutrisno RCB-LIPI
10.00 – 10.30	Tea/coffee break	
10.30 - 12.00	Lecture: Extraction of DNA from leafminers	Dr.HariSutrisno RCB-LIPI
12.00 – 13.00	Lunch break	
13.00 – 15.00	Lecture: PCR and Detection of DNA	Dr.HariSutrisno RCB-LIPI
15.00 - 15.30	Tea/coffee break	
15.30 – 17.00	Lecture: Sequencing and identification of DNA	Dr.HariSutrisno RCB-LIPI
Thursday, 10 March 2016		
Venue: Genetic Lab	SESSION 14. DNA BARCODING (continued)	
8:30 - 10:00	Practice: Extraction of DNA from leafminers	Dr.HariSutrisno/ Mr.Zein and Genetic lab team
10:00 - 10:30	Tea/coffee break	
10:30 - 12:00	Practice: Detection/PCR of DNA	Dr.HariSutrisno/ Mr.Zein and Genetic

		lab team
12:00 - 13:00	Lunch break	
13:00 - 14:30	Tea/coffee break	
15:00 - 15:30	Practice: Electrophoresis and identification of DNA	Dr.HariSutrisno/ Mr.Zein and Genetic lab team
Friday, 11 March 2016		
Venue: Seminar room	SESSION 15: EVALUATION AND CLOSING PROGARAM	
8:30 – 9:00	Post-test evaluation (non-technical)	Dr.HariSutrisno RCB-LIPI
9:00-10:30	Visit herbarium & Inacc	Paramesa & Darmawan RCB-LIPI
10:30-11.00	Tea/coffee break	
11:00-11:30	Post-test evaluation (technical)	Dr.HariSutrisno RCB-LIPI
11:30-12:00	Closing remarks related to Workshop Information related to Attachment Program Response from participants Official Closing Presentation of certificates	Dr. Hiroaki Sato Nara Women's University Dr.Soetikno S.S Dr. Hari Sutrisno RCB-LIPI Dr. R. Ubaidillah RCB-LIPI
12:00 - 13:00	Lunch break	
Saturday, 12 March 2016		
	DEPARTURE	

2. Implementation of the Program

The folowings are the detail of each session of the implementation of the program:

2.1. Season I. Opening Program and Introduction

The 20 participants from 10 different countries in Southeast Asian Region (Appendix 1; Fig.1) were welcomed by the head of Research Center for Biology-The Indonesian Institute of Science. This was followed by a short message from Dr. Lum Keng Yeang, Chairperson of APHCN-ASEANET who introduced the organization‟s mandate and project in which conducting training workshop on capacity building is one of the activities. Then, Dr. Hari Sutrisno gave the introduction and overview of the training course including the objectives, course outline and methodology. Consequently, Ms. Vany introduced each participant, resource persons and the training team.

Pre-evaluation test was given to the participants to gauge their level of knowledge on basic leafminer as well as to determine their expectations from the training workshop (Appendix 2). Also, during this session, two lectures were given. Dr. M.B. Malipatil, a lecturer from La Trobe University Melbourne Australia and Dr. Hiroaki Sato, from Nara Women University, Japan introduced the common leafminer Diptera and Lepidoptera that distribute into Indo-Austalia region.

A welcome dinner was held at Front Hall Widyasatwaloka Building, Zoology Division of Research Center Biology with the typical Indonesian cuisine and the traditional dance to entertain the participants.

2.2. Session 2. Leafminer Biology Introduction: Diptera

The aim of this season is to provide the participants with the knowledge of biology, ecology and the basic classification dipteran leafminer. During the season Dr. Malipatil gave presentation basic information of Dipetran leafminer from the biology and ecology to basic claasification of Dipteran leafminer. He also showed several important Dipteran leafminers which are common distributed to South East Asia.

2.3. Session 3. Dipteran leafminer resources on the web

In this session, Dr. Malipatil presented several resources on the web. The following links give access to a wide range of information about leafminers and their parasitoids, including images:

2.3.1. Leafminers

Polyphagous Agromyzid Leafminers [M.Malipatil and P. Ridland]

http://keys.lucidcentral.org/keys/v3/leafminers/index.htm

Crop Protection Compendium

http://www.cabicompendium.org/cpc

Martin Dempewolf's agromyzid website

http://wbd.etibioinformatics.nl/bis/agromyzidae.php

Diagnostic photomicrographs of some pest leafminers

http://www.padil.gov.au/browsePestSpecies.aspx?id=16&o=1

EPPO Diagnostic protocols, data sheets, maps & images

http://www.eppo.org/QUARANTINE/listA2.htm

Anatomical Atlas of Flies (needs Broadband)

http://www.ento.csiro.au/biology/fly/fly.html

The Anatomical Atlas was created by CSIRO Entomology to accompany an ABRSfunded identification key to fly families of Australia (on the Fly CD) and US NSF-funded research into the evolutionary history of flies.

However, the Atlas can be used as a standalone resource to accompany any fly key or as an aid for teaching fly anatomy. The atlas works both ways: users can either click on a part to discover its name, or click on a name to discover the location and shape of a part. Common synonyms for anatomical terms are available from the information button that appears when terms and structures are highlighted.

2.3.2. Leafminer Parasitoids

Lucid key to *Liriomyza* Parasitoids of Southeast Asia

by N. Fisher, R. Ubaidillah, P. Reina and J. La Salle

http://www.ento.csiro.au/science/Liriomyza_ver3/index.html

Lucid Key to the World Genera of Eulophidae Parasitoids (Hymenoptera) of Leafmining Agromyzidae (Diptera) by Placido Reina and John La Salle

http://www.ento.csiro.au/science/eulophid_key/eulophids.html

2.3.3. Book

Agromyzidae (Diptera) of Economic Importance.

K.A. Spencer 1973. Series Entomologica Volume 9 Dr W Junk B.V. – The Hague

Handbook for the identification of British Insects Diptera Agromyzidae Volume X, Part 5 (g)

K.A. Spencer 1972 Royal Entomological Society of London

C. CD ROM's

Arthropods of Economic Importance, Agromyzidae of the World

By Martin Dempewolf

ip30.eti.uva.nl/bis/agromyzidae.php

2.4. Session 4. Cabi Crop Protection Compendium

Dr. Malipatil also showed and demontrated to the participants the web site of Cabi Crop Protection Compendium: http://www.cabicompendium.org/cpc. Participants also has oppotunity to observe that web site.

2.5. Session field trips

2.5.1. Field visit to Agatho Farm (Fig.4)

Field trips were conducted basically to; (1) identify symptoms of possible leafminer-infected crops, (2) collect samples for leafminer and identification and (3) gain supplementary knowledge on different farm practices under organic and conventional farming systems. The first field trip was conducted in Agatho farm, Cisarua, where established vegetable crops: Cabbages, Cucumber, Tomato, bean, nut, lettuce, variety of herbs and other vegetables crops. Mr. Afri, a staff of Agatho Farm explained the basic vegetable production practices and management of pests and diseases. After following the presentation from the Agatho Farm, then participants were divided into two groups, A and B, each group were guided by the trainer and field assistants for filed observation. Participants were also able to collect samples showing typical leafminer symptoms (Fig. 4). This was followed by larvae selection for rearing in small rearing container to be transported and reared in the laboratory at Cibinong.

2.5.2. Field Visit to Fruit garden

The second day of field trip was held in the Fruit Garden Mekar Sari, Ciulengsi, Bogor. Mr. Deny, the head of Training and Education unit introduced how to establish the fruit garden,

the history and the purpose of the establishment of the Mekar Sari Fruit Garden. Dr. Gregory Hambali, Director of Mekar Sari Fruit Graden presented their organization‟s mandate, activities and products through a 30-minute presentation. Participants showed enthusiasts and curiosity by asking questions on the challenges faced by this farm. Participants also have opportunity to observe leafminer in several fruit trees: star trees, guava and salak. Each participant also gain local salak, guava and rambutan fruit from the farm.

2.6. Session 5. Preparation of dipteran leafminer specimens collected in the field

Preparation of specimen is the fisrt step in identification process. The preparation of specimen is started from the field during collecting. The trainer showed the sysmptom of dipetran leafminer infestation and distungish from other lefamniner and also showed how to collect the larva and pupa of dipteran leafminer in the field and also to rear them in the small rearing container. The trainer also showed how to mount the dry specimen by using the micro-insect pin. All participamt has opportunity to mount the dry specimen and observed several important characters for identification on adult specimens.

2.7. Session 6. Dissection and slide preparation of dipteran leafminer genitalia

To identify the dipteran leafminer at species level, genetalia is the most apropriate characters among the other morpholigcal caharacters. In this session, the participant was trained to improve the skill in making the preparation of genitalia slide. Only good slide that show all caharcater clearly can be used to identify species. Participants has opportunity to make slide male and female genitalia. They also learned how to distinguish male and famale genitalia and also to disect them. Participant learn also from how to mount them in canada balsam. The trainer presented and explained each caharacter of genitalia that are very improtant in identification. Indeed, experience and more exercise is needed to improve the skill, since the genitalia of lepidopteran leafminer is very small.

2.8. Session 7. Identification of dipteran leafminers

Improvement skill in identification of dipteran leafminer is the main goal in this training. The trainer provided some important dipteran leafminer, especially *Liriomyza* spp for exercise and also explained some important characters on the male genitalia and morphologicaal adult characters. Several characters whic are improtant for identification of *Liriomyza* are demonstrated by Dr. Malipatil to distinguish among species of *Liriomyza* by using a key. (**On The Fly**, The Interactive Atlas and Key to Australian Fly Families, Published by Australian

Biological Resources Study (ABRS) and Centre for, Biological Information Technology (CBIT) 2006.

2.9. Session 8. Leafminer biology introduction: Lepidoptera

The aim of this season is provide the participants with the knowledge of biology, ecology and the basic classification lepidopteran leafminer. During the season Dr. Sato gave presentation basic information of Lepidopteran leafminer from the biology and ecology to basic classification of Lepidopteran leafminer. He also showed several important Lepidoteran leafminer which are common distributed to South East Asia.

2.10. Session 9. Parasitoids associated with dipteran & lepidopteran leafminers

There are 23 species of parasitoid wasps assosiated with dipteran leafminers have been recorded from South East Asia, which they were used for the identification training. Introduction on biology of the parasitic wasps in connecting with leaf-miner were given briefly. Two hours lecturing for recognizing the methodology of collecting and preserving the parasitoids wasps including Malise traping, yellow traping, swept netting and rearing the hosts were domenstrated. The next four hours, recognizing of the main characters of the parasitoids wasps which is found on lepdiopteran leafminer, especially, *Liriomyza* spp, were given directly under the stero microscope. The two hours in the end of the training, the participants were given the specimens for practizing identifion of the parasitods foud from West Java and Central Java. Test for identification of the parasitoids were given in the closing identification training.

2.11. Session 10. Dissection and preparation of lepidopteran leafminer genitalia

To identify the lepidopteran leafminer at species level, genetalia is the most apropriate characters among the other morpholigcal caharacters. In this session, the participant was trained to improve the skill in making the preparation of genitalia slide. Only good slide that show all caharcater clearly can be used to identify species. Participants has opportunity to make slide male and female genitalia. They also leaned how to distinguish male and famale genitalia and also to disect them. Participant learn also from how to maccerate the abdomen by using 10% KOH, cleaning, staing by using clhorazol balack, series dehidrating by using ethanol, arranging the position o valva, to mounting in canada balsam. The trainer presented and explained each caharacter of genitalia that are very improtant in identification. Indeed,

experience and more exercise is needed to improve the skill, since the genitalia of lepidopteran leafminer is very small.

2.12. Session 11. Preparation of wings

Wing venation is important characters to identify at certain level, most of families in Lepidptera was distinguish based on wing venation. In this session, participant has opportunity to remove the wing from the body, to remove the scale from the wing, to stain the wing by using Fuchsin acid and observed each venation both forwing and hindwing. The trainer also presented several improatnt character of wing venation to identify some species of leaf miner.

2.13. Session 12. Identification of lepidopteran leafminers

Improvement skill of identification is the main goal in this training. The trainer provided some important lepidopteran leafminer for exercise and also explained some important characters on the male genitalia. Several characters such as shape of valva, transtila, vinculum, aedeaghus, tegumen and cornuti are demonstrated by Dr. Sato to distinguish among species of Japanese *Phyllonorycter* spp. (Gracillariidae) associated with oaks (Quercus spp., Fagaceae). Participants has also opportunity to observe and identify the provided specimens by using guid line book.

2.14. Session 13. Preparation slide of larva

This session trained the participant to improve their skill and tehir knowledge in recognizing several characters that are very improtant in identifiction of ledipopteran leafminer. Participant has oportunity to make slide by using provided larvae. Each participant make slides from different type of feeding larvae both feeding and sucking types. Participant also able to distinguish between Hypognathous and Prognathous head, and recognize the cahetoxy system and several types of abdominal legs.

2.15. Session 14. DNA Barcoding

DNA barcoding technique for identification leafminer was provided to improve the knowledge and skill participants in identifiction based on molecular data. In this training, several procedur in routin molecular work was presented such as Collecting insect pests for molecular identification, DNA Extraction, PCR, Electrophoresis, DNA Sequencing, Alignment data, Analysis data for identification. Paarticipnt also has oportunate to identify their own specimen by using specific primer of *Liriomyza*. Most participant able to identify *Liriomyza trifoli* and *L. huedobrensis* based on the length of PCR Product.

2.16. Session 15. Evaluation and closing program

The overall evaluation showed that majority of the participants achieved their expectations from the training workshop (Fig 5). This outstanding rating was reflected from the very satisfactory results of their post-evaluation test. Seventhy perscent (70%) got confidence level in identifiction of *Liriomyza* and 30% indicated fairly confidence level. The training team including the resource persons and logistics also received high percentage of overall rating (4 – Good to 5- Excellent) from the participants (Appendix 2).

Appendix 1: The list of Organizer, Resources persons, and Participants

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Regional Training Coordinator

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Mr. M. Rofik Sofyan – Local Training Coordinator – Technical (MZB)

Dr. Awit Suwito – Local Training Coordinator – Administrative (MZB)

Ms. Gina Andriana – Local Training Secretary (MZB)

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Appendix 2. Results of Pre-test & Post test

1. Results of Pre-test

Essay (index Falls (0) – Right (1))	0	0.3	0.5	0.6	0.8	1
1. Name 3 species of leafminers?	0	8	0	5	0	7
2. What do you want to learn from this workshop?	0	0	0	0	0	20
3. What can leafminers have an impact on?	8	1	2	0	0	9
4. What is the DNA barcode?	8	1	5	0	0	6
5. Give two morphological characters which distinguish insects from the other arthropods!	8	0	2	1	0	9
6. Give a common name(s) for each of Lepidoptera, Hymenoptera, Diptera and Coleoptera!	11	0	3	0	1	5

Multiple Choice	1= No conf.	2=Fairly	3= Confident
7. How confident are you that you could collect leafminer specimens in the field?	7	9	4
8. How confident are you that you could give advice on leafminers to a co-worker in your own country?	11	8	1
9. How confident are you that you could prepare a slide mount of a leafminer specimen?	11	7	2

10. Do you know how to get help to identify leafminer specimens? (Please circle)
    (1) No idea how (2) Have some idea
    (3) Good idea how
    (4) Know well how to get help

4 (Have some idea)
8 (Good idea how)
8 (Know well how to get help)

11. Overall, how would you rate your knowledge of leafminers (✓ below)

	1	2	3	4	5
Ecology / Biology	10	8	2	0	0
Morphology	10	8	2	0	0
Ability to identify species	13	3	4	0	0
How to extract from samples collected in the Field	8	7	5	0	0
How to preserve samples for DNA extraction	15	2	1	2	0

2. Results of Post-test

Non technical-Post test

1. Lectures and laboratory sessions

Using the rating scale below, circle the response that represents your honest opinion

Lectures session	**1**	**2**	**3**	**4**	**5**
Activity met the stated learning objectives	0	0	1	10	9
Content was presented in a well-paced, structured format	0	1	3	6	10
Content was relevant to my job	0	1	3	6	10
Content was presented in a well-paced, structured format	0	0	1	6	10
Content was pitched at the right level for the audience	0	0	4	8	8
An appropriate mix of techniques were used to convey the content (e.g. case study, group work, discussion,lecture, etc)	0	0	0	10	10
Notes/materials were good quality and will be Useful for my job	0	0	2	5	13
I can immediately apply my learning to my job	0	0	5	10	9
I had ample opportunity to network with Colleagues	0	0	2	10	8

Laboatory session

▯ Activity met the stated learning objectives	0	0	1	9	10
▯ Content was relevant to my job	0	1	4	7	8
▯ Content was presented in a well-paced, structured Format	0	0	3	12	5
▯ Content was pitched at the right level for the audience	0	0	4	7	10
▯ An appropriate mix of techniques were used to convey the content (e.g. group work, discussion, lecture, etc)	0	0	1	8	11
▯ Notes/materials were good quality and will be useful for my job	0	1	1	7	11
▯ I can immediately apply my learning to my job	0	1	6	4	9
▯ I had ample opportunity to network with colleagues	0	0	1	11	8

2. Main/Principal Speaker(s) and Facilitator(s) Evaluation

Using the rating scale below, circle the response that represents your honest opinion.

Main/Principal Speaker Name: Dr. Malipatil	**1**	**2**	**3**	**4**	**5**
▯ Knowledge of the subject matter	0	0	0	3	17
▯ Approachable and able to answer questions	0	0	0	5	15
▯ Engaging and able to maintain audience interest	0	0	0	5	15
▯ Showed respect for the audience's skills and experience	0	0	0	3	17
▯ Used a variety of facilitation/presentation techniques	0	0	1	3	16
▯ Punctual and kept agreed timeframes	0	0	2	4	14
▯ Well organized and prepared	0	0	1	5	14

Main/Principal Speaker Name: Dr. Sato					
▯ Knowledge of the subject matter	0	0	0	4	16
▯ Approachable and able to answer questions	0	0	1	5	14
▯ Engaging and able to maintain audience interest	0	0	0	12	8

Showed respect for the audience's skills and experience	0	0	1	6	13
Used a variety of facilitation/presentation techniques	0	0	1	9	10
Punctual and kept agreed timeframes	0	0	3	9	8
Well organized and prepared	0	0	1	5	14

Main/Principal Speaker Name: Dr. Hari

Knowledge of the subject matter	0	0	0	4	16
Approachable and able to answer questions	0	0	0	4	16
Engaging and able to maintain audience interest	0	0	0	5	15
Showed respect for the audience's skills and experience	0	0	0	4	16
Used a variety of facilitation/presentation techniques	0	0	1	4	14
Punctual and kept agreed timeframes	0	0	2	7	11
Well organized and prepared	0	0	1	1	16

Main/Principal Speaker Name: Dr. Rosichon

Knowledge of the subject matter	0	0	0	5	15
Approachable and able to answer questions	0	0	1	7	12
Engaging and able to maintain audience interest	0	0	0	11	9
Showed respect for the audience's skills and experience	0	0	0	8	12
Used a variety of facilitation/presentation techniques	0	0	1	10	9
Punctual and kept agreed timeframes	0	0	2	7	11
Well organized and prepared	0	0	2	7	11

3. This activity might be more useful if:

- More activities on Identification, collection and preparation of specimens and field survey : 2 answers
- To give more time for practical with more equipment. Samples should be limited per group as some groups no. of samples double – 7 answers

- To provide a bigger screen TV/monitor with high resolution and appropriate height
- Training manual should be organized and no missing/delayed in giving notes
- If all participants have the same level of knowledge in leafminers
- Sample preparation for PCR should be done 1 day earlier as waiting time was too long.
- Follow up training with lepidopteran or dipteran leafminers only – 2 answers
- Internet access always available daily
- The tables used for practicals (microscopes) should be strong so that not shaking!
- Advance training on leafminers for the same participants

4. General arrangements – Logistics, field trip, etc

Please comment on the effectiveness/efficiency of arrangements for this workshop.

- Very effective, good arrangement for logistics and field trip – 12 answers
- Very good arrangement, please repeat this workshop – 2 answers
- Arrival at the airport – should be using a big LIPI logo
- Food at LIPI should have more varieties and must cater for other cuisines, e.g. Chinese, western, etc. and more in quantity – 2 answers
- Information about the field trip should be given as clear as possible, i.e. crops grown in the area, acreage, production, etc.

4.1. For future training courses, what changes would you recommend to logistical arrangements?

- Identification of leaf-miners using DNA sequences – 2 answers
- To have a good connection of internet – 2 answers
- A big LIPI logo should be used on the arrival gate of the airport
- Food should be prepared as buffet style.
- Participants should be requested to bring hand-lenses
- Given more time for lunch break

Overall, how would you rate this workshop? (below)	**3=** Satisfactory	**4=** Good	**5=** Excellent
• Accommodation	3	10	7
• Workshop venue/training venue facilities	7	13	
• Travel arrangements	1	5	14

• Field Trip	1	5	14
• Food/refreshments	6	10	4

4.2. Any other comments?

- Gaining new knowledge
- Well organized training – should be repeated in the future
- Training materials should be given before departure of participants so that they can read in advance. Also software to be used in the training.
- LIPI Staff very kind, cooperative and helpful
- Food not enough quantity, more vegetables.
- Especially for PCR practical, lecture on this should be given first before doing practical.

Technical Post Test

Multiple Choice	1	2	3	4
1. How confident are you to indentify (Liriomyza spp.) specimens in your country? (Please circle) (1) no confident, (2) fairly confident, (3) confdent	0	6	14	0
2. How confident are you that you could give advice on leafminers to a co-worker in your own country? (Please circle) (1) no confident, (2) fairly confident, (3) confident	1	8	11	0
3. How confident are you that you could prepare a slide of leafminers specimens?(Please circle) (1) no confident, (2) fairly confident, (3) confident	1	9	10	0
4. Do you know how to get help to identify leafminer specimens? (Please crcle) (1) No idea how (2) Have some idea (3) Good idea how (4) Know well how to get help	0	4	8	8
5. Do you have enough facilities to conduct the DNA Barcode for leafminer specimens? (Please circle) (1) complete facilities (2) PCR only (3) PCR & sequencing machines (4) no facility	2	9	4	5

Eassay (index Falls (0) – Right (1))	**0**	**0.3**	**0.5**	**0.6**	**0.8**	**1**
1. What is the best collection method for parasitoids leaf-miner, please give the advantages ?	6	0	1	0	2	11
2. Do you know, how many species of parasitoids associated with dipteran leaf-miner in South-East Asia?	14	0	0	0	3	3
3. How many species species of parasitoids associated with dipteran leaf-miner in your country, Please write the species name and their family belong to ?	8	0	7	0	2	3
4. Do you know how many species of parasitoids of lepidopteran leaf-miner in your country, , Please write the species name and their family belong to ?	9	0	6	0	2	3
5. What the main characteristic of Figitidae (*Gronotoma* sp), Eulophidae (*Closteroceros* sp) and Braconidae (*Opius* sp) ?	11	2	3	0	2	2
6. What is the main characters of *Hemiptarsenus varicornis*?	11	0	1	0	0	7
7. How many type DNA extraction method, Please give the advantages and disadvantages of each method?	6	0	2	0	1	11
8. Please state the procedure to identify *Liriomyza* by using specific primer?	14	0	0	0	1	5
9. How you can distinguish among species of *Liriomyza* based on multiplex PCR product	11	0	0	0	0	9
10. How to check the PCR product?	14	0	0	0	0	6
11. Please state non destruction method!	10	2	0	0	0	8
12. When you observe leafminers, what dou you pay attention to?	9	1	0	0	1	9
13. There are three types of female genitalia						

Question						
in the Lepidoptera. Explain differences among three the type	16	0	0	0	0	4
14. Explain hypermetamorphosis in the Gracillariidae?	14	0	0	0	0	6
15. What are 1 to 4 structures in figure?	14	6	0	0	0	0
16. When you identify the species from morphology of the male genitalia, which characters do you pay attention to?	10	4	3	0	0	3
17. Please give five name species of *Liriomyza* leafminer commonly found in Southeast Asian countries?	0	1	0	2	2	15
18. Please give five name species of *Liriomyza* leafminer commonly found in Southeast Asian countries?	4	0	1	0	4	11
19. How you collect adult flies and larvae of leafminer specimens in the field?	2	1	1	1	3	12
20. What character diagnostic for Diptera?	3	6	1	1	1	8

Yes/No

Question	Yes	No
21. If you selected as a candidate for the advance training as attachment program, are you willing to work on leafminers and their parasitoids? (Please cross) ( ) Yes, ( ) N0	12	5

Figure 1. Photo Group

Figure 2. Gala Dinner

Figure 3. Trainig Lab

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Figure 4. Field Trip

Figure 5. Post-Test

Attachment 6

2nd Progress Report on NETWORKING AND INSTITUTIONALISATION

TAXONOMIC CAPACITY BUILDING TO SUPPORT MARKET ACCESS FOR AGRICULTURAL TRADE IN THE ASEAN REGION (AGF/CRO/11/007/REG)

Prepared by

F.W. Chan, S.S. Sastroutomo and K. Y. Lum

April 2016

2nd Progress Report for JAIF

NETWORKING AND INSTITUTIONALISATION

Objectives of this component include information sharing, information dissemination and mainstreaming and institutionalisation of information through networking. The information gained through networking and exchange of information becomes embedded and enters the common knowledge domain of respective institutions and NPPOs of countries concerned.

Mainstreaming and institutionalisation of information is achieved through the various activities in the project; training and capacity building workshops, attachment programs with experts, engagement through the project website, online tools and services and other project activities.

The project website provides a platform to host tools and services e.g. expert register databases, online diagnostic tools, pests and diseases information etc.

To raise awareness and disseminate information, the use of offline and online media will be used. Marketing and promotional materials and collaterals may come in the form of flyers, posters, brochures, online web feeds, or e–newsletters which will be produced and distributed.

Latest project activities

The section on project activities is now completed. This section hosts news and updates on the latest project activities and other activities related to the project. From the first activity comprising of the project inception and steering committee meeting to the latest training workshops on the plant viruses and leafminers, the project activities section now hosts all the very latest updates, images, documents and outputs related to the aforesaid activities.

The section was revamped with a better layout featuring images followed by update and news on the activity. The document section is at the bottom of the project activity news body and displays all outputs related to that activity. The title and format of the document can be seen but the link to download is only accessible after logging in to the project website.

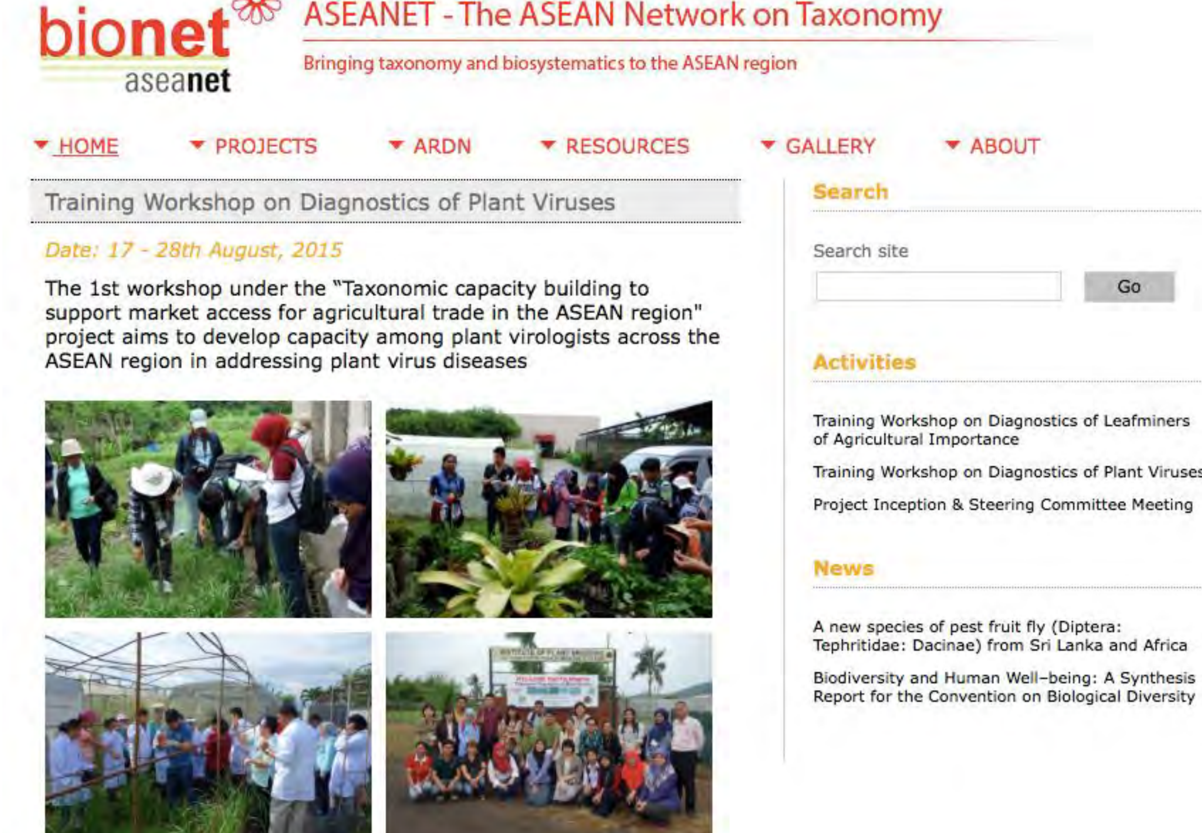

Fig. 1 – Section on project activities with images

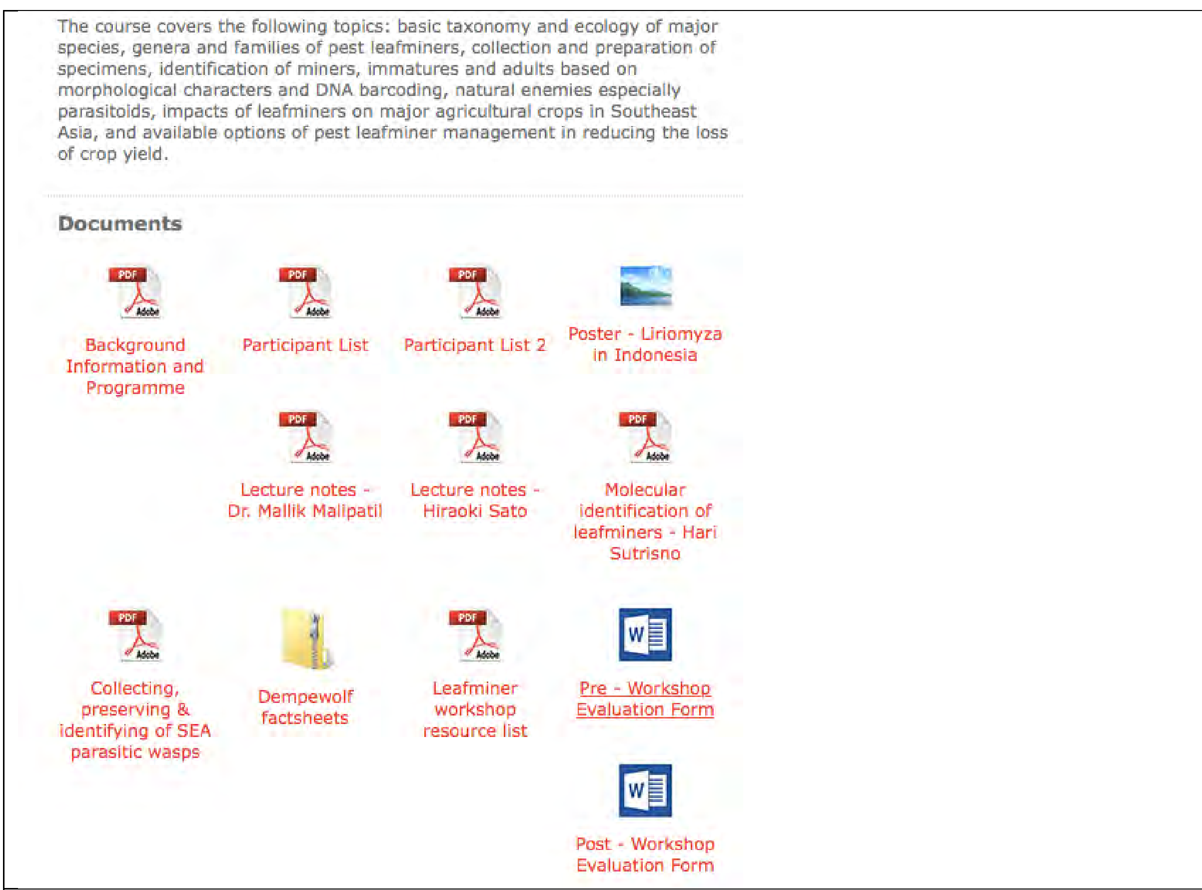

Fig. 2 – Documents related to project activities

Content management

The content management system built into the website to manage content in the website is now completed. This system allows content to be uploaded, edited or removed. It also allows content to be tagged for listing and searching. There is a feature in the content management system for content to be archived and not removed if that is the preferred option. Archiving means content is saved in the system but not displayed, and can be later retrieved if needed.

The content management system is not available for public and regular users of the website as it is meant for only the administrator and the content team. It is accessed and viewable after logging in to the system.

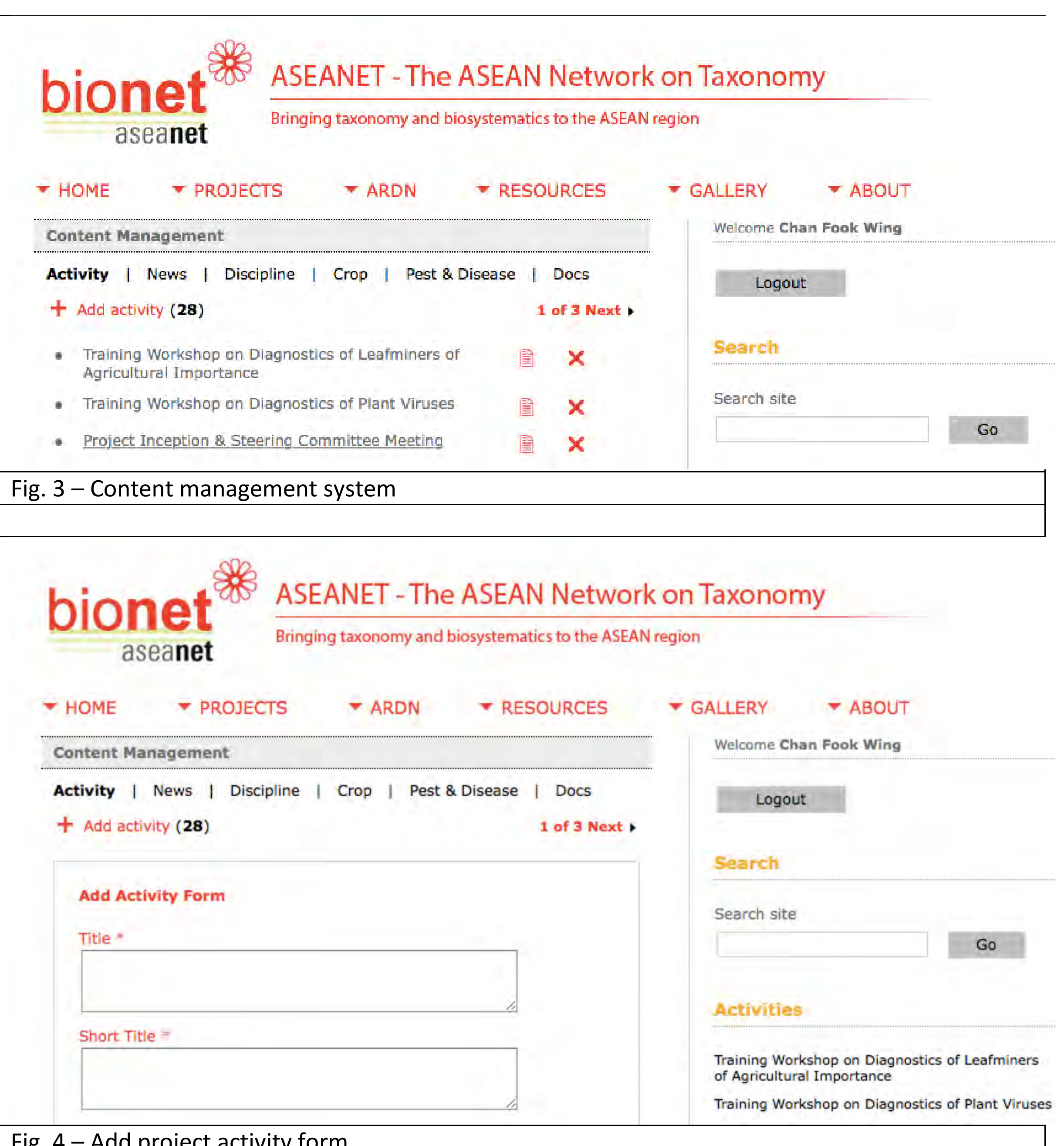

Fig. 3 – Content management system

Fig. 4 – Add project activity form

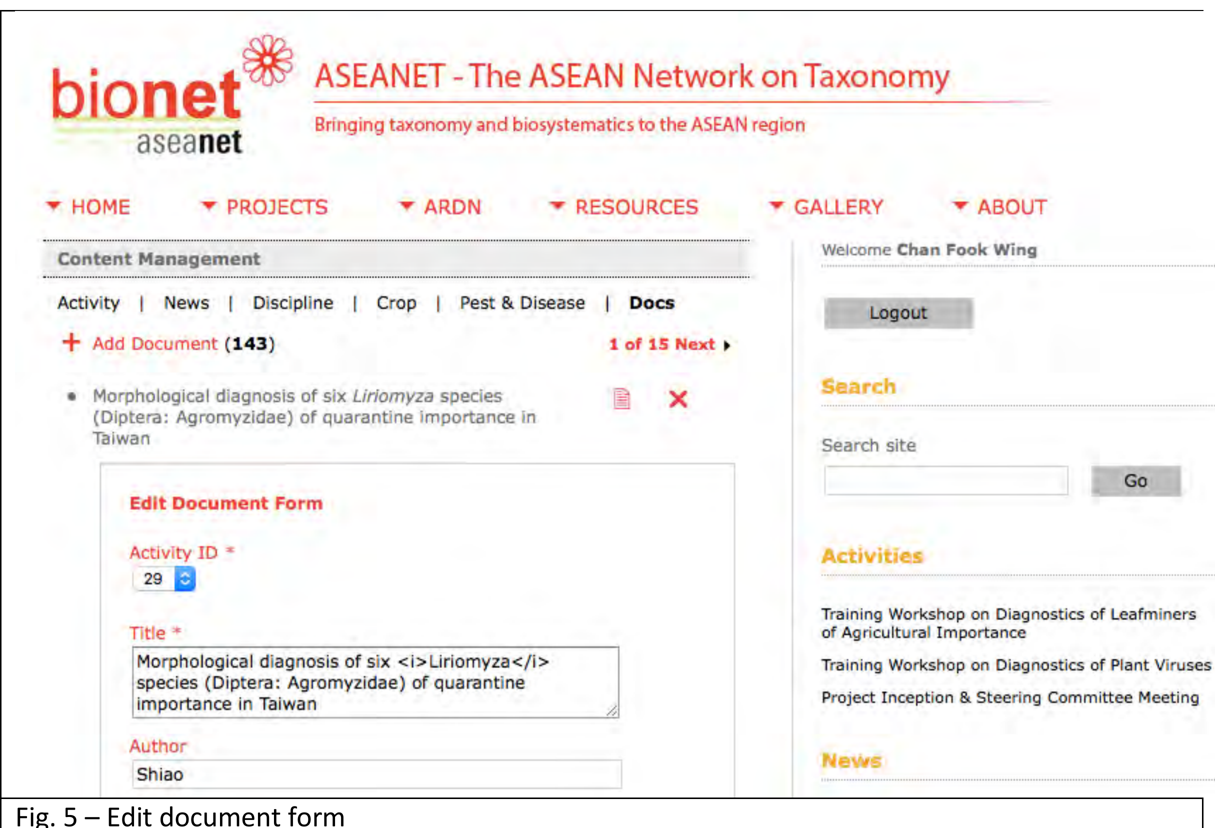

Fig. 5 – Edit document form

Project download section

The project download section is meant for project partners to access for the purpose of downloading documents and other outputs related to activities carried out during the project. The documents come in different formats depending on the output produced during the project activity. All formats are catered for and different icons are used for the different format to provide visual differentiation when viewing documents.

The logins for this section will be provided to project partners for them to be able to access the resources contained therein.

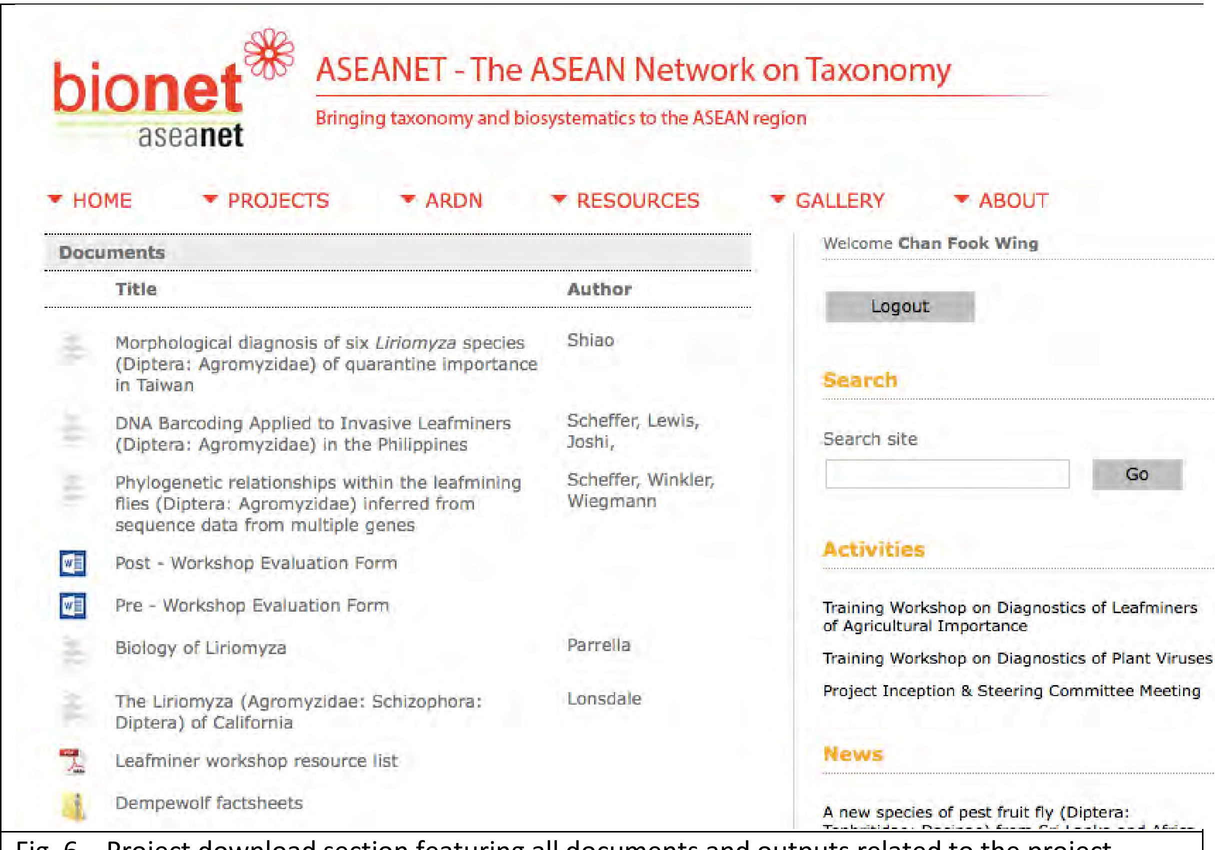

Fig. 6 – Project download section featuring all documents and outputs related to the project

Pictures Gallery

The pictures gallery holds images from various project activities. Each set of images are separated according to project activity in thumbnail format in rows of three. Images are large enough for viewing at a quick glance in the gallery. Clicking on each image brings up a pop-up window displaying a larger view of the image.

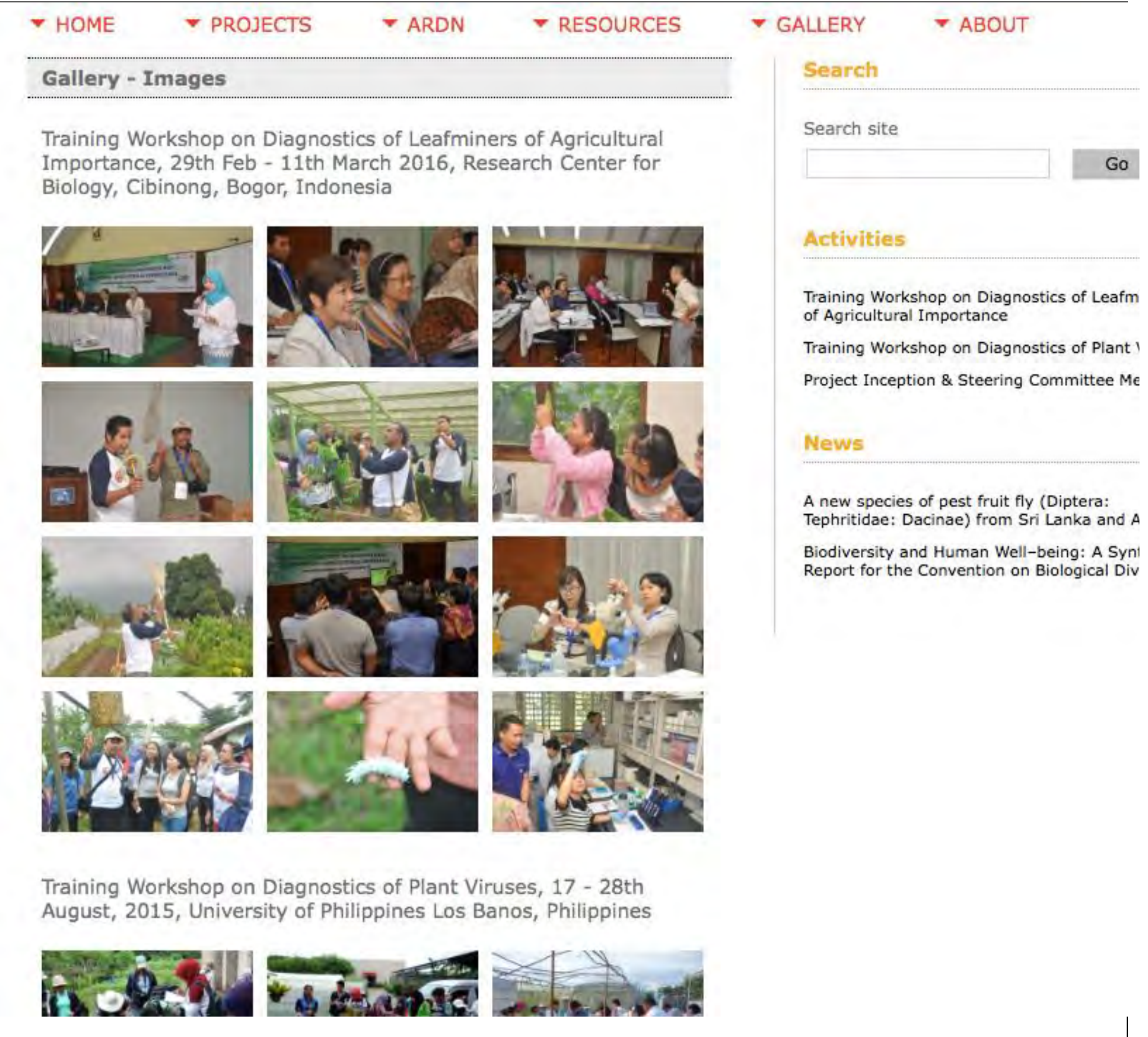

Fig. 7 – Image gallery

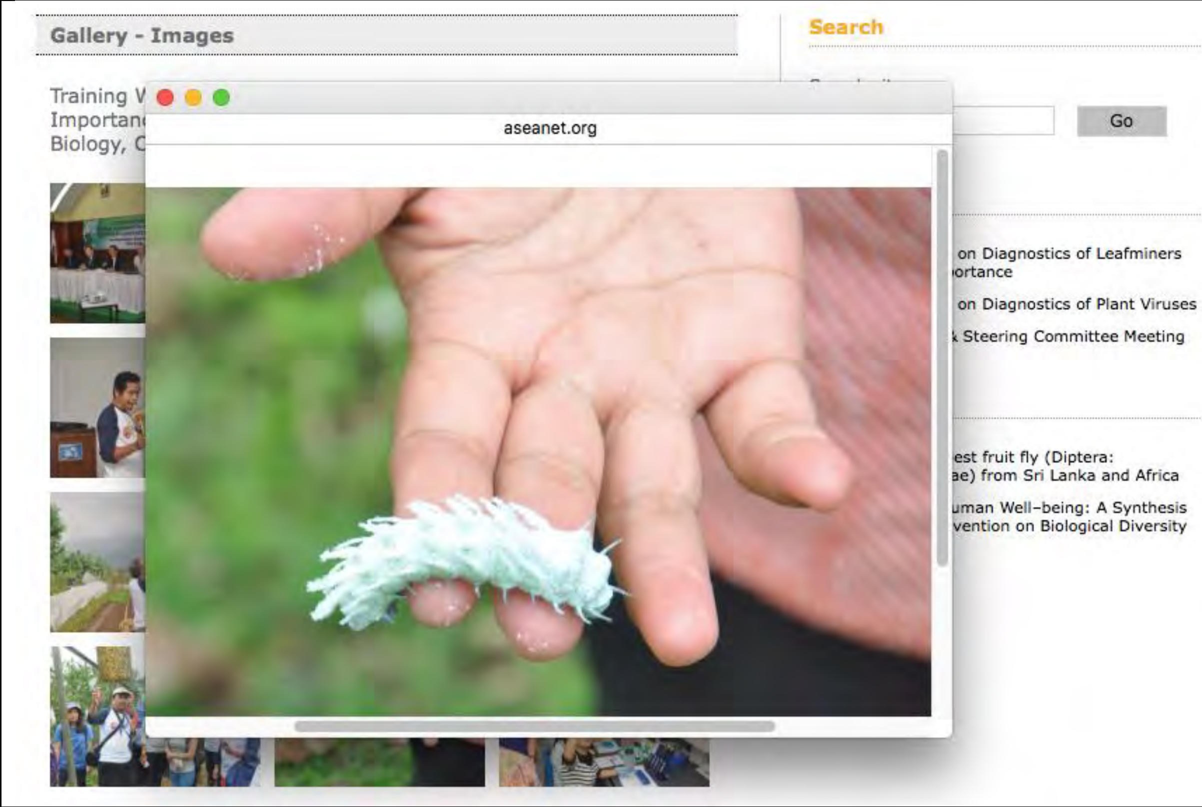

Fig. 8 – Image pop – up window

Expert Register

The basic expert register module is now ready and the register is populated with data from the previous database of contacts comprising participants and resource persons from capacity building workshops on taxonomy carried out over a number of years. Many of the individual experts belong to institutions that provide identification and diagnostic services and facilities for plant pests and diseases. The experts and diagnostic laboratories are mainly located in the region i.e. Southeast Asia.

The register provides a quick way for users to obtain information on experts and institutions (laboratories and universities) that are able to provide expertise in plant pests and diseases identification and diagnosis. Contact details for experts are available for users to refer to.

Further work needs to be carried out to enable users to communicate with experts more efficiently; with tools to facilitate real – time messaging and chats, perhaps even voice and conversations, the ability to exchange and send images and videos, experts status and readiness to respond to requests for assistance, mechanism and process to acknowledge requests for help and confirmation of assistance and attendance.

More importantly the register list must be updated to reflect the current status of experts and institutions willing and able to provide their expertise when requests are made from users.

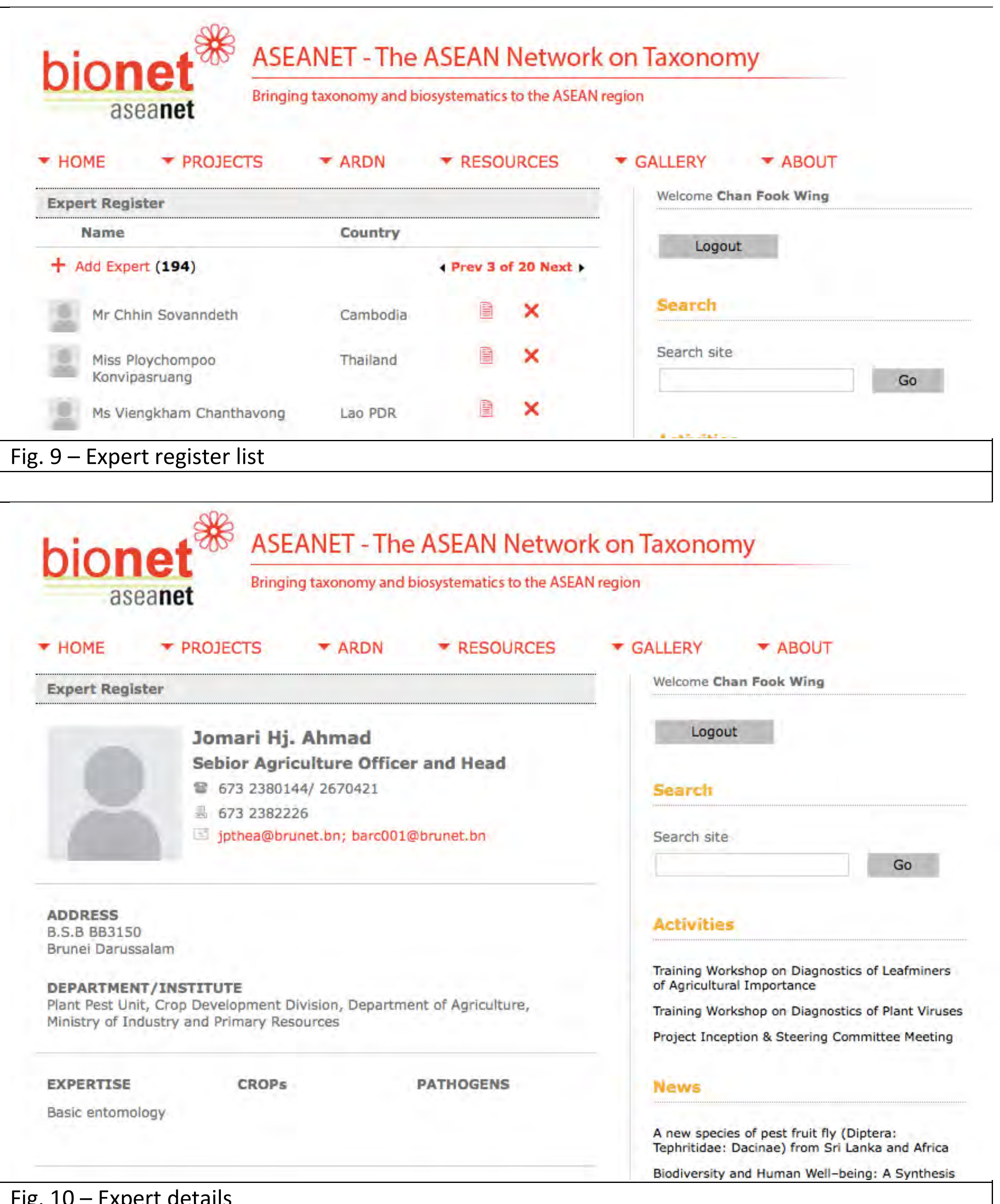

Fig. 9 – Expert register list

Fig. 10 – Expert details

Mobile version of the expert register

The mobile version of the register has been developed and is compatible on major mobile platforms, allowing for quick access and reference to the register from smartphones and mobile devices.

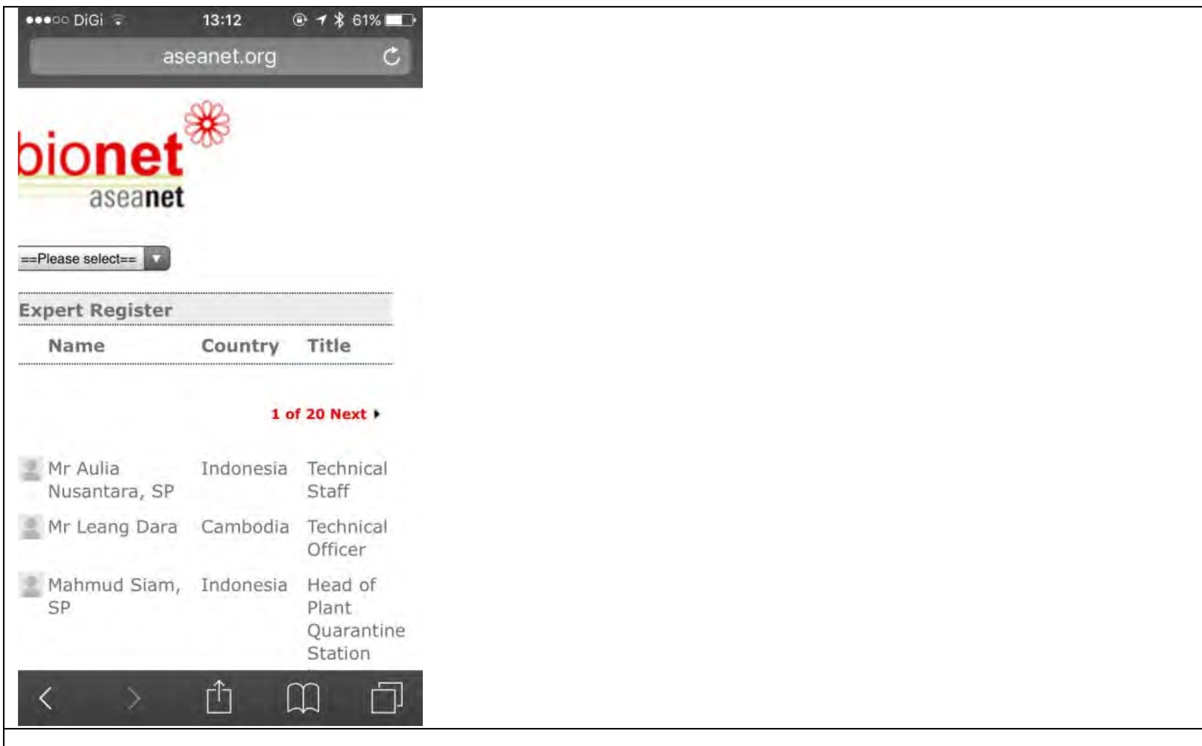

Fig. 11 – Mobile version of the expert register list

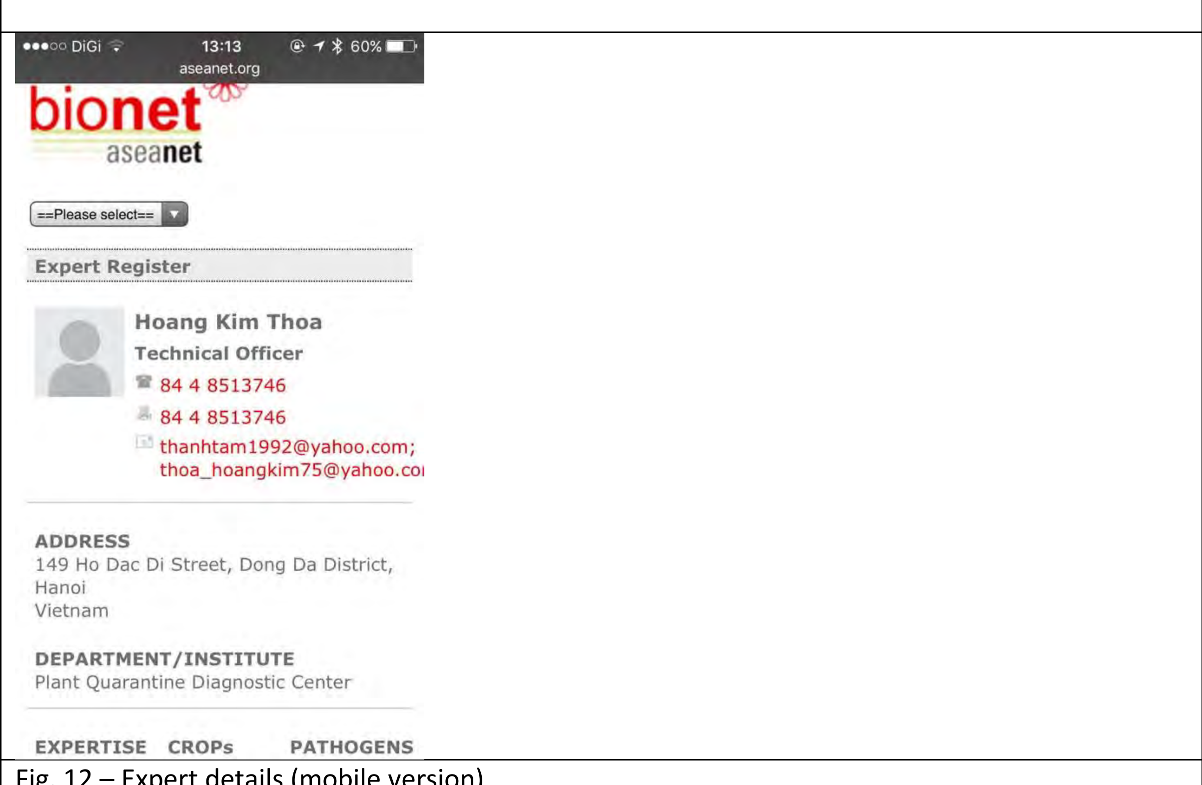

Fig. 12 – Expert details (mobile version)

On – going work

Clearinghouse

The clearinghouse feature, first proposed and mooted under the ASEAN Regional Diagnostic Network, has now moved into the ASEANET website. The clearinghouse mechanism, where samples are sent to the facility based in Malaysia where initial identification and recording of specimens are undertaken and later consigned to experts drawn from the expert register where identification and diagnosis is made, remains. With the move to ASEANET, initiatives can be drawn up under the current JAIF project and other capacity building activities to engage countries in the region and encourage the use of the clearinghouse facility.

E-application for diagnostic services, online resources and tools

Under the e-application for diagnostic services feature, the electronic sample submission form for the clearinghouse facility is currently being migrated into ASEANET. To improve the service, the e-forms for sample submission will be tracked and status updates be given to senders/clients to allow them to monitor the progress and status of their submissions. Status and tracking will be carried out via the website and email.

To further support identification and diagnosis and provide updated tools, online identification and diagnostic tools / keys can be built into ASEANET. Some examples could be remote microscopy, mobile microscopes and online web-based pests and diseases identification and diagnostic tools.

Pests and diseases information database and image databank

The pests and disease information for earlier samples sent through the clearinghouse facility is in the process of migration from ARDN into ASEANET. The sample database is being migrated in parallel and the information categories would be re-worked to include image fields to accommodate the build-out of an image databank. The image databank and pests and diseases database is crucial to support taxonomy and identification and diagnosis expertise for the region.

ATTACHMENT 7

FINANCIAL SUMMARY

Project Title : Taxonomic capacity building to support market access for agricultural trade in the ASEAN Region
Proponent/Implementing Agency : ASEAN Plant Health Cooperation Network (APHCN)
Duration/Period : 01/10/2015 to 30/04/2016

Budget Line	Approved Budget (X)	Actual Expenses (Y)	Balance (X-Y)
FUND RECEIVED ON (08/06/2015)			**267,706.23**
FUND RECEIVED ON (26/11/2015)			**267,706.23**
I. PROGRAMME/ACTIVITY COST			
Component 1. Training and Capacity Building			
1.1. Organize training workshops in the ASEAN countries in 3 taxonomic subjects for 14 days			
Airfare	**53,100.00**	**25,579.73**	**27,520.27**
Consultant/Expert	**135,998.00**	**64,276.59**	**71,721.41**
DSA	**222,684.00**	**126,892.84**	**95,791.16**
Training Workshop	**17,400.00**	**13,270.05**	**4,129.95**
1.2. Attachment programs in Japan for 3 taxonomic subjects	**305,640.00**	**63,723.36**	**241,916.64**
Sub Total for Training and Capacity Building (I)	**734,822.00**	**293,742.58**	**441,079.42**
Component 2. Networking and Institutionalization			
C.1 **Developed database on taxonomic expertise**	**15,000.00**	**252.60**	**14,747.40**
C.2 **Develop website on ARDN**	**7,500.00**	**3,750.00**	**3,750.00**
C.3 **Develop promotional materials**	**5,000.00**	**127.66**	**4,872.34**
C.4 **Maintaining and updating website**	**12,600.00**	**7,000.00**	**5,600.00**
Sub Total for Networking and Institutionalization (II)	**40,100.00**	**11,130.26**	**28,969.74**
Component 3. Management and Coordination			
Project Organization and Coordination	**16,800.00**	**8,400.00**	**8,400.00**
Project Monitoring & Implementation	**14,617.00**	**1,318.50**	**13,298.50**
Project Inception Meeting & Project Final Meeting	**17,640.00**	**5,509.13**	**12,130.87**
Sub Total for Management and Coordination (III)	**49,057.00**	**15,227.63**	**33,829.37**
SUB TOTAL OF PROGRAMME COST (I + II + III)	**823,979.00**	**320,100.46**	**503,878.54**
Contingency 10% (IV)	**68,375.10**	**105.15**	**68,269.95**
TOTAL (I + II + III + IV)	**892,354.10**	**320,205.62**	**572,148.48**

Total Fund Received		535,412.46
Total Expenditure		320,205.62
- Previous reported expenditure	123,918.33	
- Current expenditure	196,287.29	
Balance		215,206.84

FINANCIAL REPORT

Project Title : Taxonomic capacity building to support market access for agricultural trade in the ASEAN Region
Proponent/Implementing Agency : ASEAN Plant Health Cooperation Network (APHCN)
Duration/Period : 01/10/2015 to 30/04/2016

Budget Line	Description	Reference Number	Approved Remainng Budget (X)	Actual Expenses in Other Currency		Actual Expenses (Y)	Balance (X-Y)
	FUND RECEIVED ON (08/06/2015)			**MYR**	**1,000,257.56**		**267,706.23**
	FUND RECEIVED ON (26/11/2015)			**MYR**	**1,143,694.56**		**267,706.23**
		060					
I. PROGRAMME/ACTIVITY COST							
Component 1. Training and Capacity Building							
1.1. Organize training workshops in the ASEAN countries in 3 taxonomic subjects for 14 days							
Airfare							
A.1	International Consultant (Japan)		8,482.12	JPY	99,120.00	987.92	7,494.20
A.2	Regional Consultant		3,141.86	MYR	398.00	106.52	3,035.34
A.3	Participants from ASEAN		28,911.72	MYR	44,541.60	11,920.99	16,990.73
Total expenses for Airfare			**40,535.70**			**13,015.43**	**27,520.27**
Consultant/Expert							
A.4	Project Manager (Consultant)	-	72,000.00			24,000.00	48,000.00
A.5	International Consultant (Japan)		4,568.00				4,568.00
A.6	Regional Consultant		23,725.41			4,572.00	19,153.41
Total expenses for Consultant/Expert			**100,293.41**			**28,572.00**	**71,721.41**
DSA							
A.7	International Consultant (Japan)		8,475.35			2,710.00	5,765.35
A.8	Regional Consultant		15,999.00			4,065.00	11,934.00
A.9	Participants from ASEAN		141,505.81			63,414.00	78,091.81
Total expenses for DSA			**165,980.16**			**70,189.00**	**95,791.16**
Training Workshop							
A.10	Venue and equipment		3,430.00	IDR	31,000,000.00	2,296.30	1,133.70
A.11	Training Material: laboratory expenses		4,258.50	MYR	3,885.39	3,759.73	498.77
				IDR	36,718,000.00		
A.12	Training Material: Photo copy of course materials		1,500.00	IDR	4,415,500.00	327.07	1,172.93
A.13	Field trips for sample collections		1,944.18	IDR	11,215,000.00	830.74	1,113.44

FINANCIAL REPORT

Project Title : Taxonomic capacity building to support market access for agricultural trade in the ASEAN Region
Proponent/Implementing Agency : ASEAN Plant Health Cooperation Network (APHCN)
Duration/Period : 01/10/2015 to 30/04/2016

Budget Line	Description	Reference Number	Approved Remainng Budget (X)	Actual Expenses in Other Currency		Actual Expenses (Y)	Balance (X-Y)
A.14	Communications		211.11				211.11
Total expenses for Training Workshop			**11,343.79**			**7,213.84**	**4,129.95**
1.2. Attachment programs in Japan for 3 taxonomic subjects							
B.1	Airfare for Participants (Selected Scientists) from ASEAN		27,000.00	MYR	14,350.90	3,840.84	23,159.16
B.2	International Consultant (Japan)		36,000.00			9,000.00	27,000.00
B.3	DSA for Participants (Selected Scientists) from ASEAN (in Tokyo, Nara, Sapporo)		197,640.00			42,456.00	155,184.00
B.4	Venue and equipment		18,000.00			6,120.00	11,880.00
B.5	Training Material: laboratory expenses		12,600.00			540.00	12,060.00
B.6	Training Material: Photo copy of course materials		1,800.00			-	1,800.00
B.7	Communications		3,600.00	MYR	99.12	26.53	3,573.47
B.8	Honorarium for technicians		9,000.00			1,740.00	7,260.00
Total expenses for Attachment programs in Japan for 3 taxonomic subjects			**305,640.00**			**63,723.36**	**12,633.47**
Sub Total for Training and Capacity Building (I)			**623,793.06**			**182,713.64**	**211,796.26**
Component 2. Networking and Institutionalization							
	Developed database on taxonomic expertise						
C.1	Supplies and services – database software and coordination/communication		14,747.40	MYR		-	14,747.40
	Develop website on ARDN						
C.2	Supplies and services – web development, testing and coordination/ communication	-	7,500.00			3,750.00	3,750.00
	Develop promotional materials						
C.3	Supplies and materials – Printing leaflets & flyers (1,000 copies)		4,872.34	MYR		-	4,872.34
	Maintaining and updating website						
C.4	Project Administrative- US$ 700 x 18 months for 1 person (Website maintenance officer)	-	9,800.00			4,200.00	5,600.00
Sub Total for Networking and Institutionalization (II)			**36,919.74**			**7,950.00**	**28,969.74**

FINANCIAL REPORT

Project Title : Taxonomic capacity building to support market access for agricultural trade in the ASEAN Region
Proponent/Implementing Agency : ASEAN Plant Health Cooperation Network (APHCN)
Duration/Period : 01/10/2015 to 30/04/2016

Budget Line	Description	Reference Number	Approved Remainng Budget (X)	Actual Expenses in Other Currency		Actual Expenses (Y)	Balance (X-Y)
Component 3. Management and Coordination							
Project Organization and Coordination							
D.1	Project Administrative/Finance Assistant	-	12,600.00			4,200.00	8,400.00
Total expenses for Project Organization and Coordination			**12,600.00**			**4,200.00**	**8,400.00**
Project Monitoring & Implementation							
D.2	airfare (to ASEAN)		1,400.00	MYR	369.90	99.00	1,301.00
D.3	DSA in Jakarta		1,897.00			1,219.50	677.50
D.4	DSA in Manila		1,638.00			-	1,638.00
D.5	airfare (to Japan)		6,000.00			-	6,000.00
D.6	DSA in Tokyo		2,240.00			-	2,240.00
D.7	DSA in other city (elsewhere in Japan)		1,442.00			-	1,442.00
Total expenses for Project Monitoring & Implementation			**14,617.00**			**1,318.50**	**13,298.50**
Project Inception Meeting & Project Final Meeting							
D.8	Airfare		7,028.58	MYR		-	7,028.58
D.9	DSA (Kuala Lumpur)		2,444.06	MYR		-	2,444.06
D.10	Meeting Package		840.00	MYR		-	840.00
D.11	Secretariat Supplies		818.23	MYR		-	818.23
D.12	Preparing Project Reports		1,000.00			-	1,000.00
Total expenses for Project Inception Meeting & Project Final Meeting			**12,130.87**			-	**12,130.87**
Sub Total for Management and Coordination (III)			**39,347.87**			**5,518.50**	**33,829.37**
SUB TOTAL OF PROGRAMME COST (I + II + III)			**700,060.68**			**196,182.13**	**274,595.38**
Contingency 10% (IV)			**68,375.10**	MYR	198.60	105.15	**68,269.95**
				USD	52.00		
TOTAL (I + II + III + IV)			**768,435.78**			**196,287.29**	**342,865.32**

FINANCIAL REPORT

Project Title : Taxonomic capacity building to support market access for agricultural trade in the ASEAN Region
Proponent/Implementing Agency : ASEAN Plant Health Cooperation Network (APHCN)
Duration/Period : 01/10/2015 to 30/04/2016

Budget Line	Description	Reference Number	Approved Remainng Budget (X)	Actual Expenses in Other Currency	Actual Expenses (Y)	Balance (X-Y)

Note: All in USD.
Exchange Rate: US$1.00 @ RM3.7364 @ IDR13,500.