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

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(Indonesia)

Organized by:

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

bionet aseanet
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)

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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

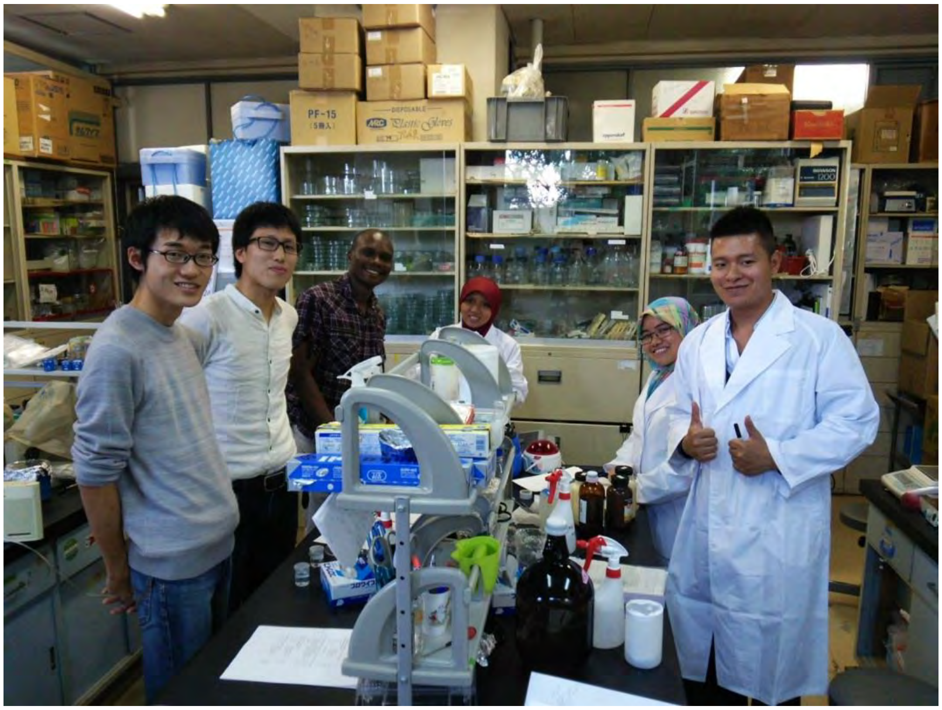


Fig. 1 First laboratory activity, preparation of buffer for ELISA test and virus transmission

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).

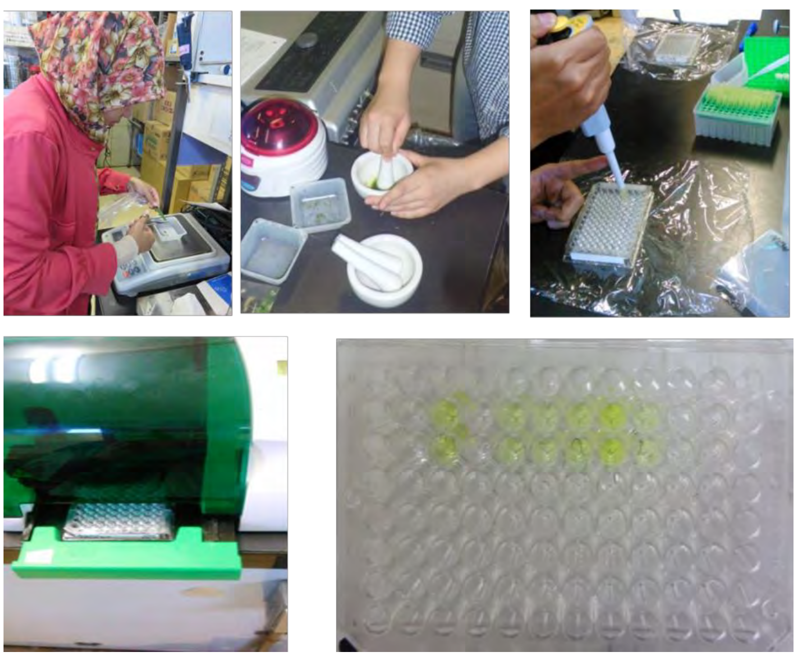


Fig. 3 Potyvirus detection using indirect ELISA method

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.

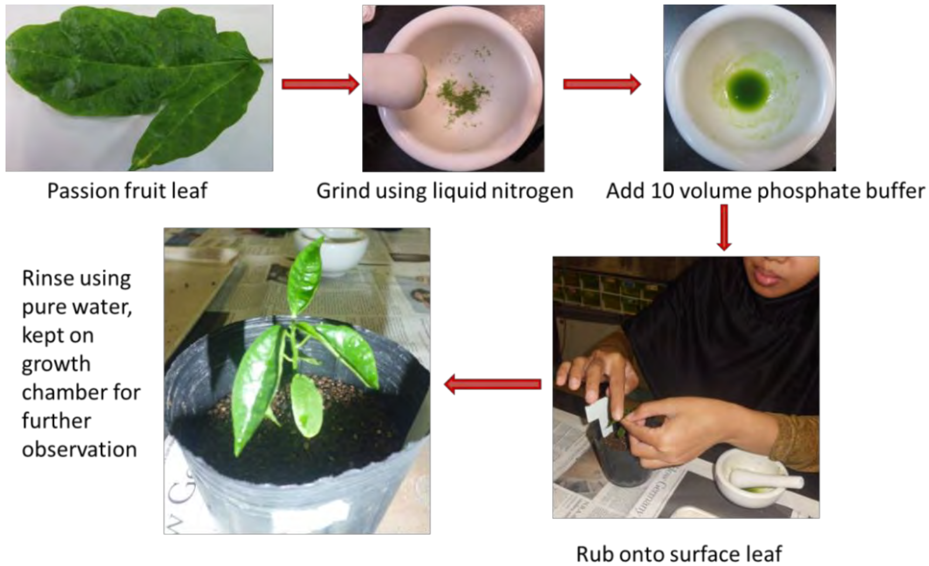


Fig. 5 Process of virus transmission using mechanical inoculation

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.

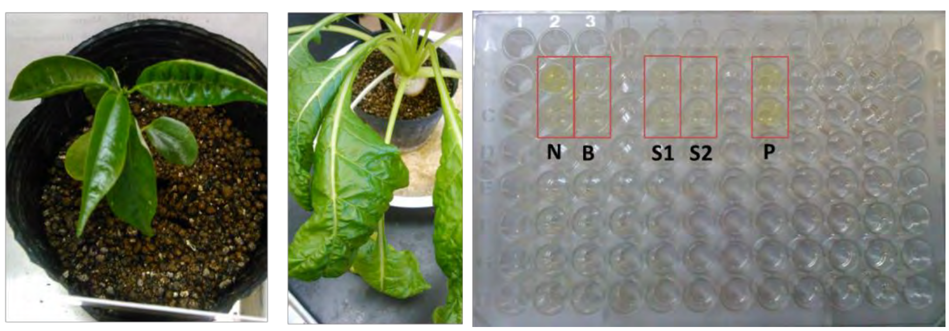


Fig. 6 Symptom observation after 2 weeks and evaluate with indirect ELISA for potyvirus

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

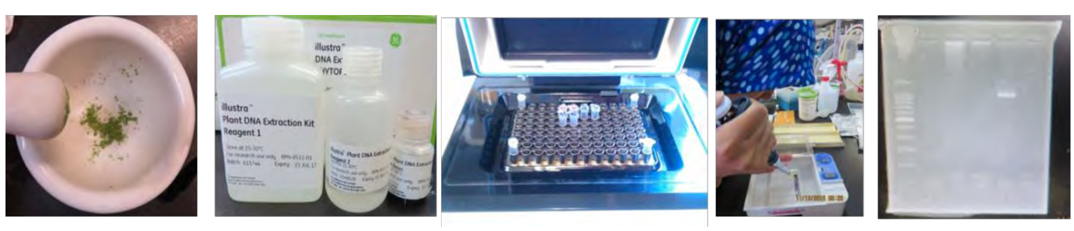


Fig 7. DNA extraction, PCR, and DNA visualization on gel agarose

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.

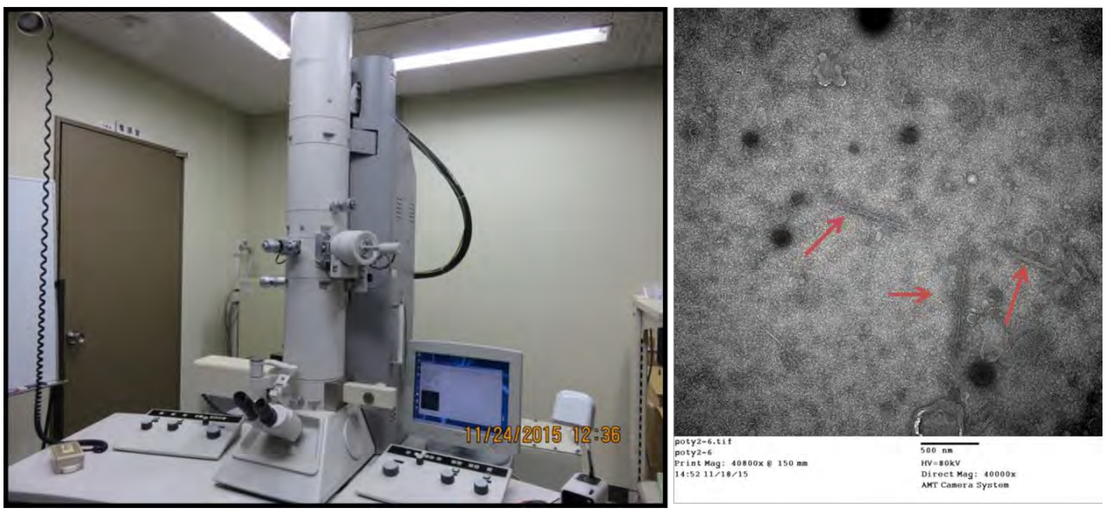
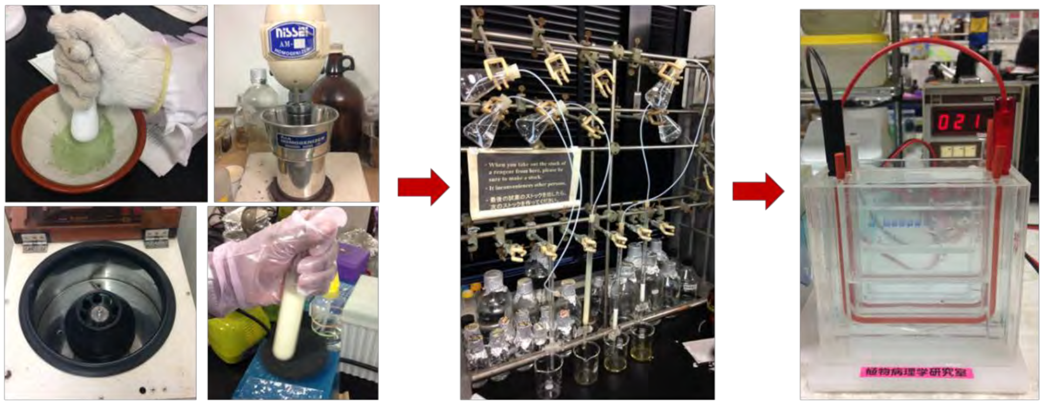


Fig 11. Observation of potyvirus under Electron Microscope (EM)

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



Pulverized and homogenized

Absorption dsRNA in cellulose powder as a filter

Electrophoresis in polyacrylamide gel

Fig 12. dsRNA extraction from cucurbits infected by CMV

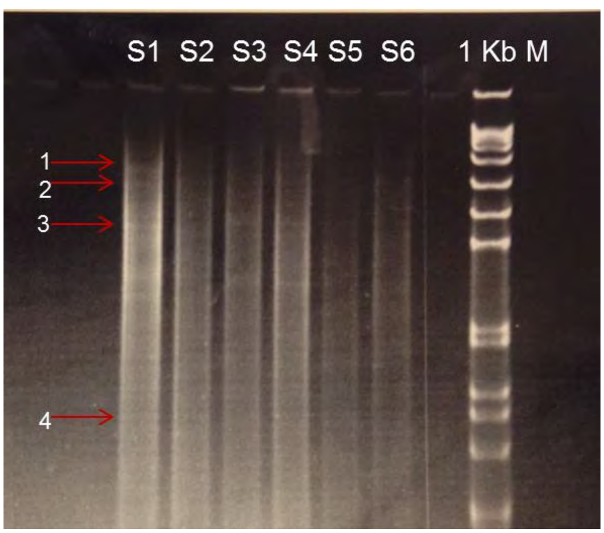


Fig 13. CMV dsRNA banding pattern have 3 main genome and one sub genome encoded coat protein

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.

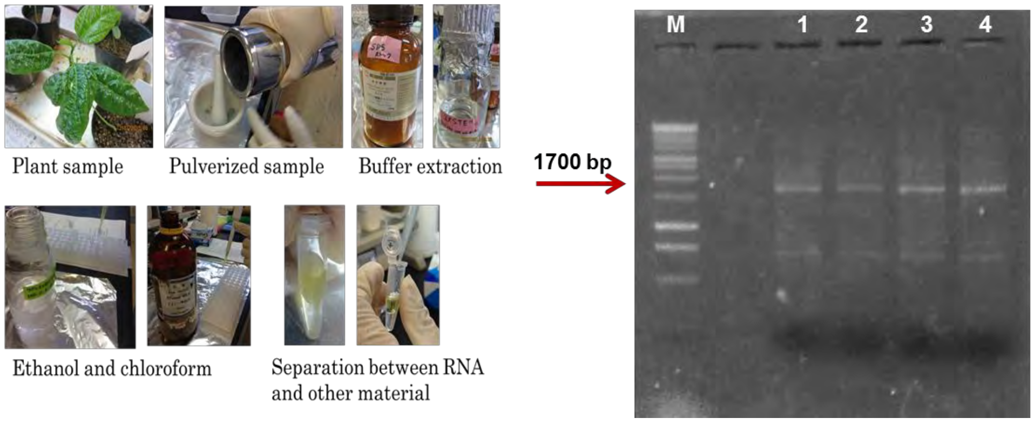


Fig 14. Extraction of RNA potyvirus from Passion fruit, and the result showed DNA band in 1700 bp compatible with DNA of potyvirus

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.

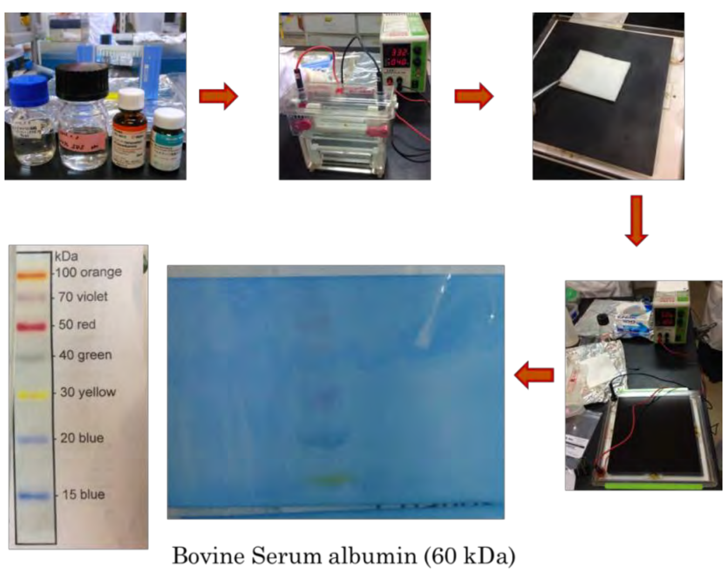


Fig 15. Process of SDS-PAGE and Western blot for sample of bovine serum albumin (BSA)

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 (35ºC) or low (15ºC) 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.

LIST OF APPENDIXES

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.

Appendix 2

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

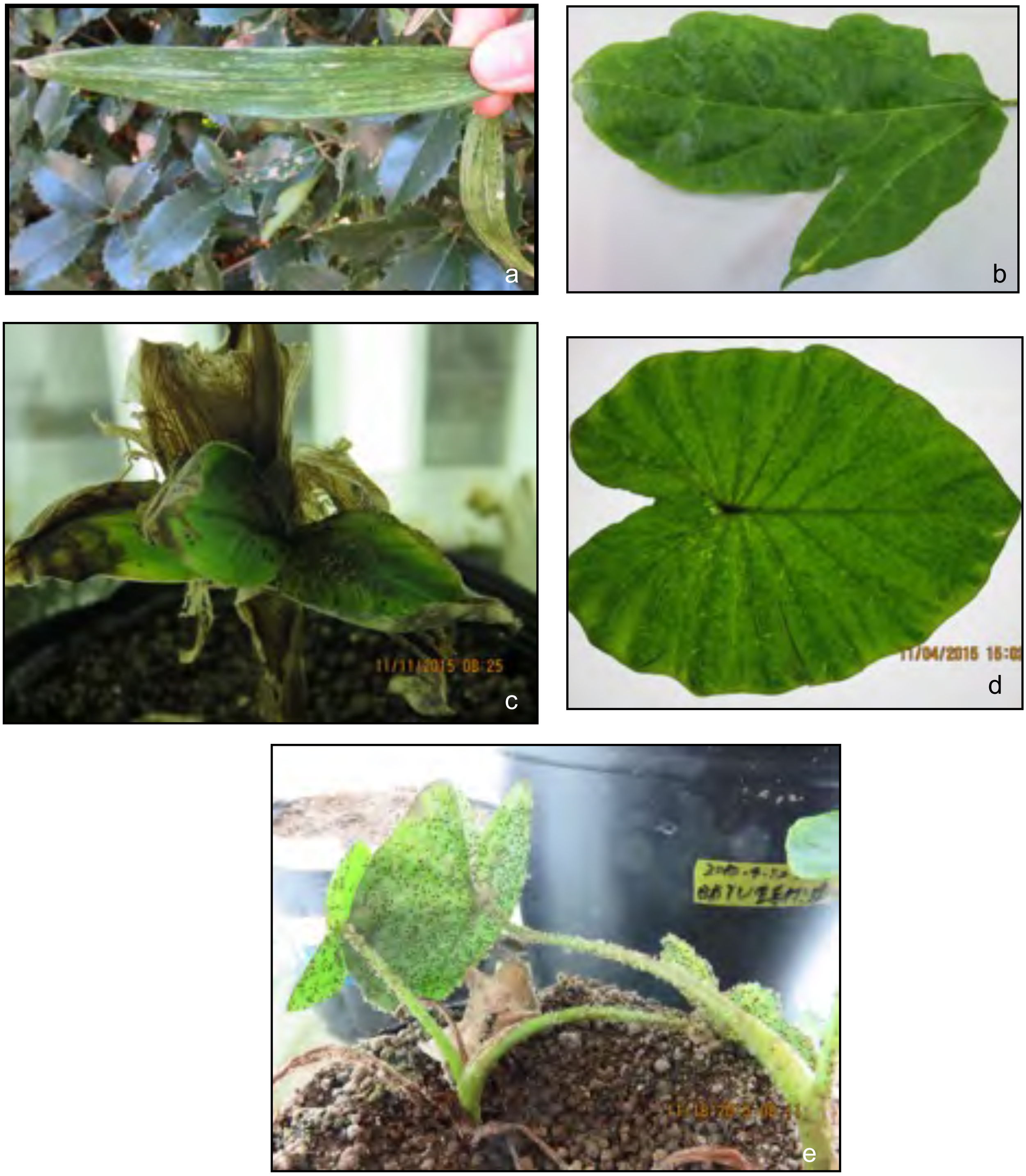
Xgal (2% 5-bromo-4-chloro-3-indole-β-D-galactoside): 5 mL

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^{o}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.

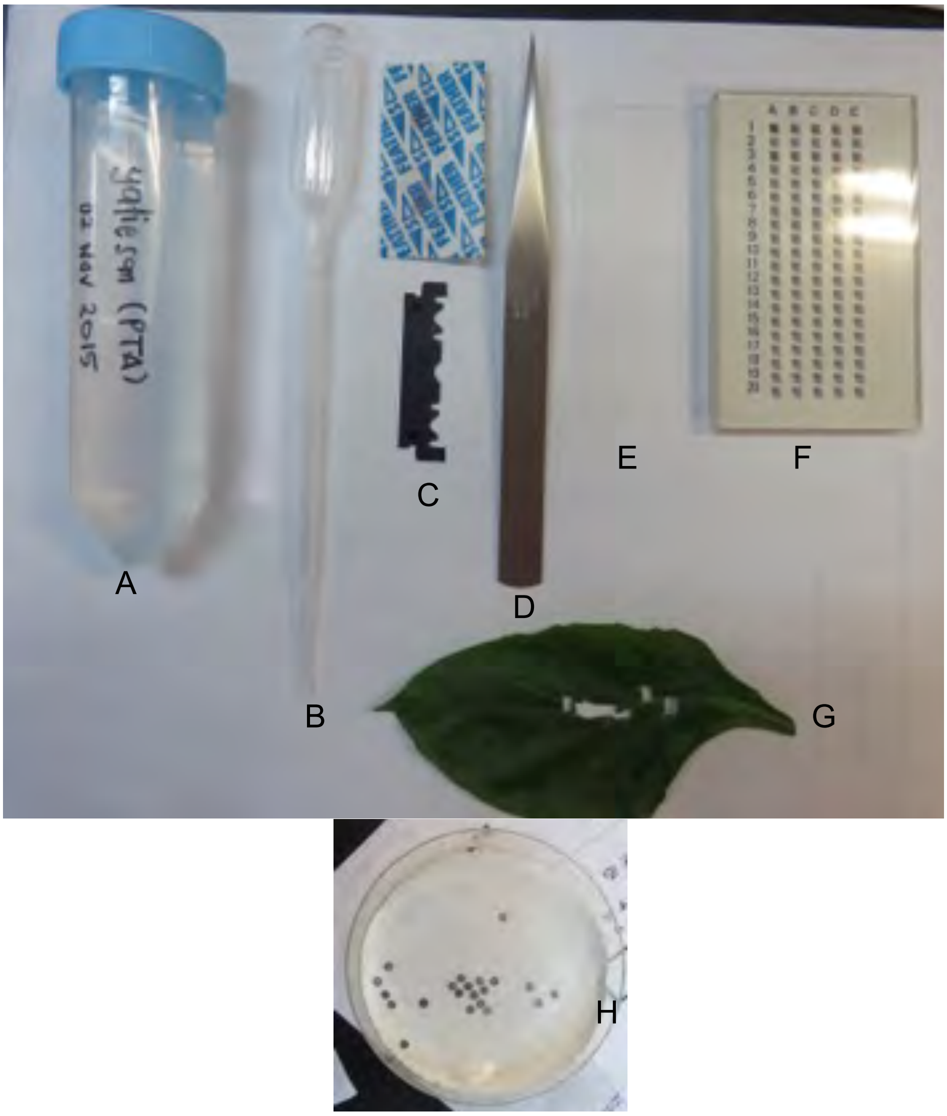


Figure 1: Materials used for the preparation of samples. (A) PTA reagent; (B) plastic dropper; (C) blades; (D) forcep; (E) glass slide; (F) disc grid; (G) leaf sample; (H) copper disc.

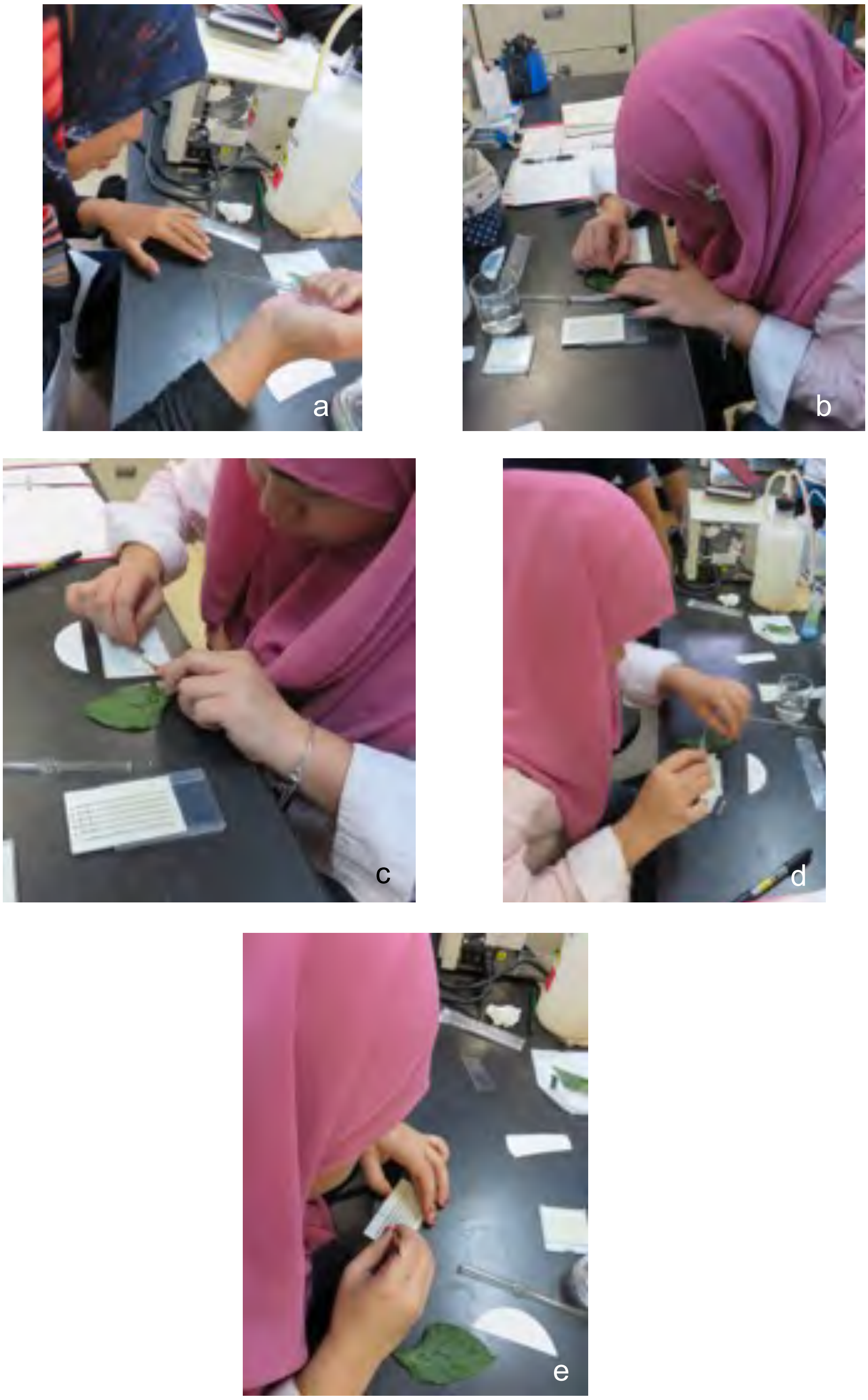


Figure 2: Preparation of sample using deep method.
(a) One drop of PTA reagent was dropped on the glass slide; (b) a small size of leaf sample was cut off including portion of vein; (c) the leaf was crushed and mixed with PTA reagent; (d) the copper disc was dried using filter paper; (e) the disc was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

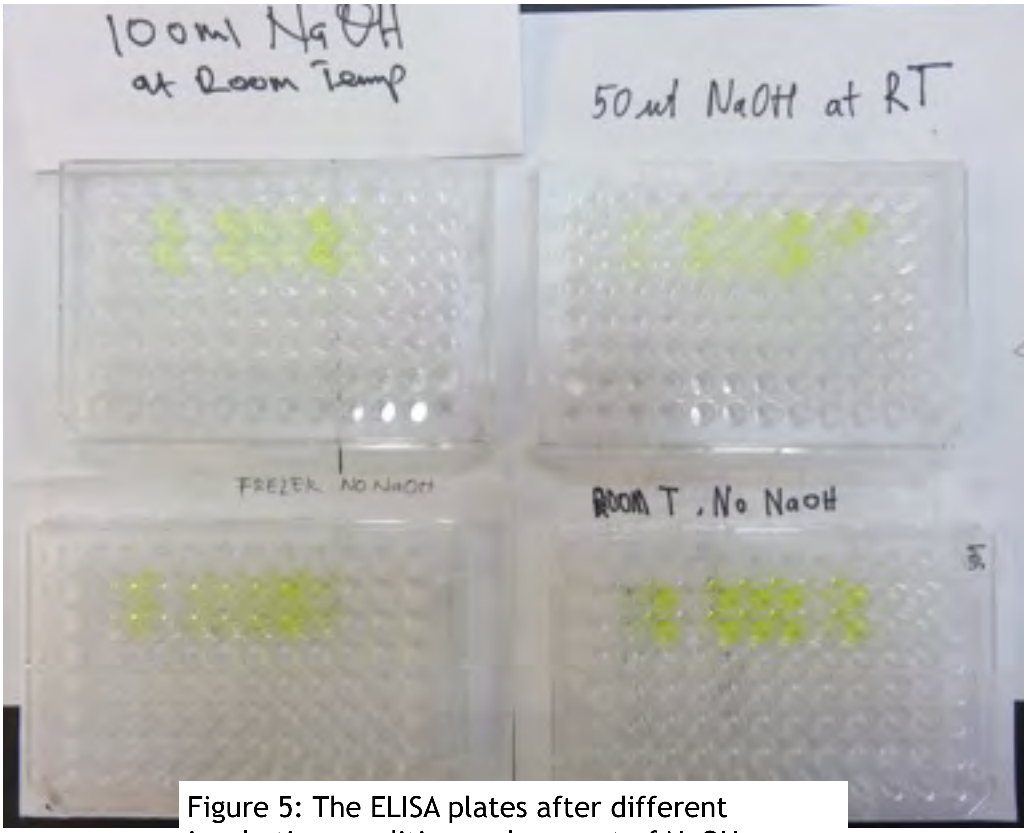


Figure 5: The ELISA plates after different incubation condition and amount of NaOH added.

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).

<u>a) Darsheen Mosaic Virus (DSMV)</u>

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.

<u>b) Colocasia Bobine Disease Virus (CBDV)</u>

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.

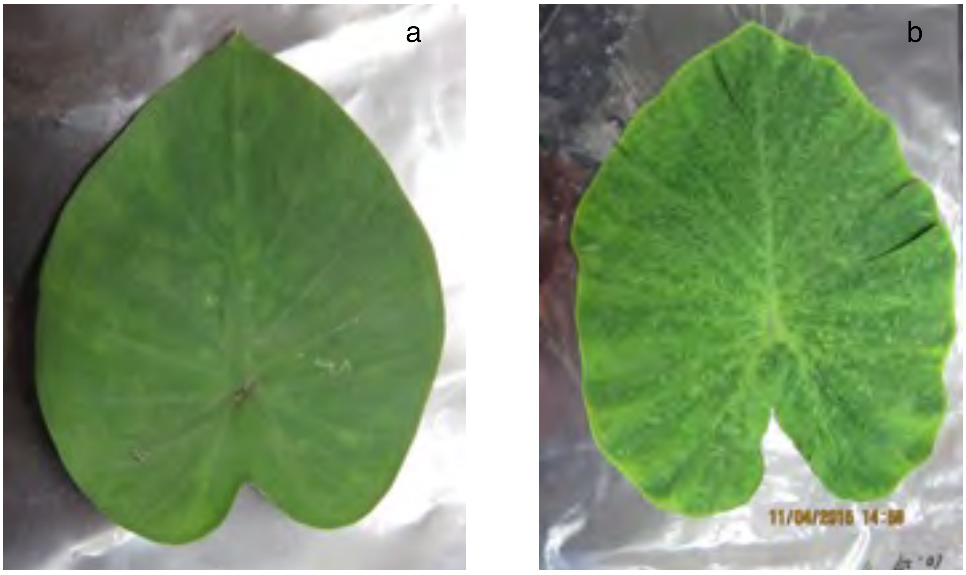

Figure 1:
Infected taro leaves showed mosaic symptom. (a) T808 T254P;
(b) T706 T19

Microplane Layout

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

Notes

	Negative control (healthy taro leaves)
	Positive control
	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) Potyviruses diseases

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

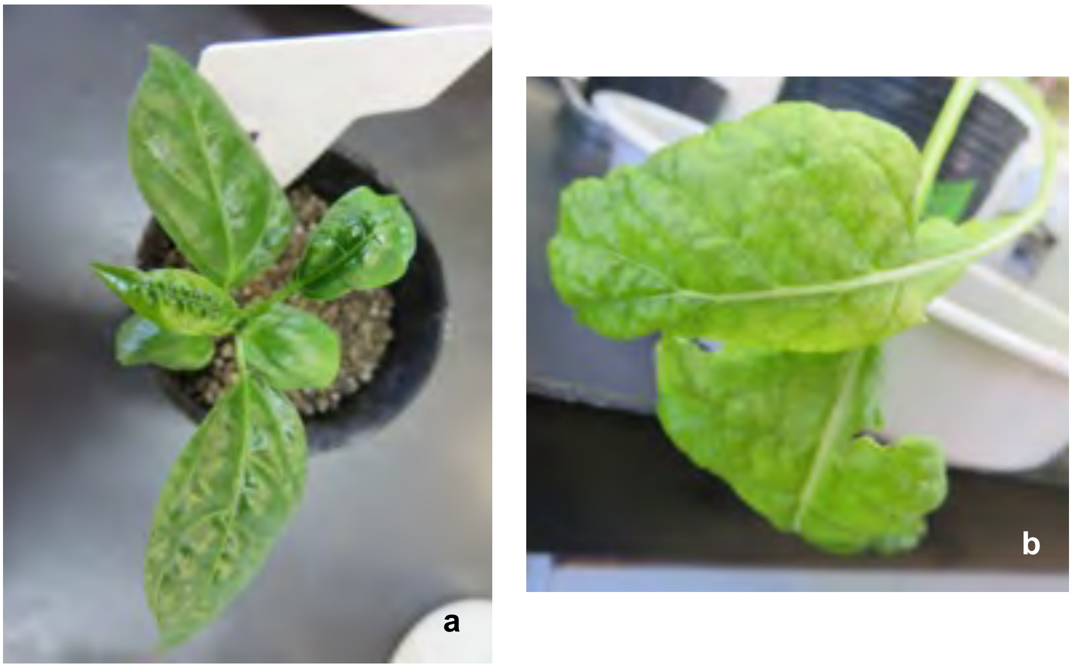

Figure 4: Plant samples used for ELISA test. (a) passion fruit inoculated with unknown virus; (b) reddish inoculated virus.

Microplate Layout

Note:

1	2	3	4	5	6	7	8	9	10	11	12
				S1	S2						
				S1	S2						

	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^{o}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

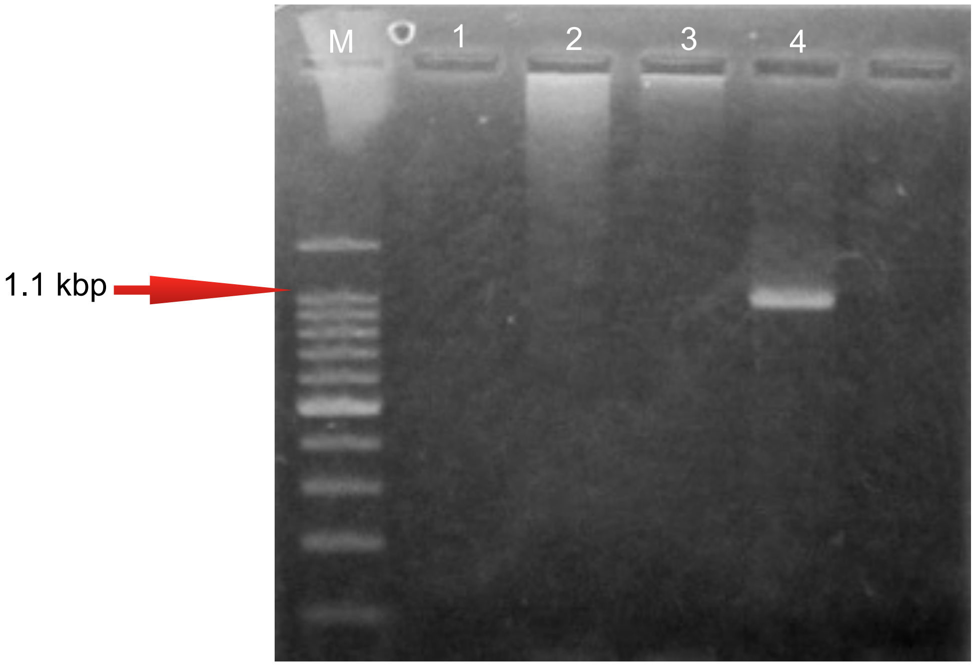

Figure 3: Gel electrophoresis of DNA extracted from banana leaves. The PCR products were analysed on 2% gel.

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.

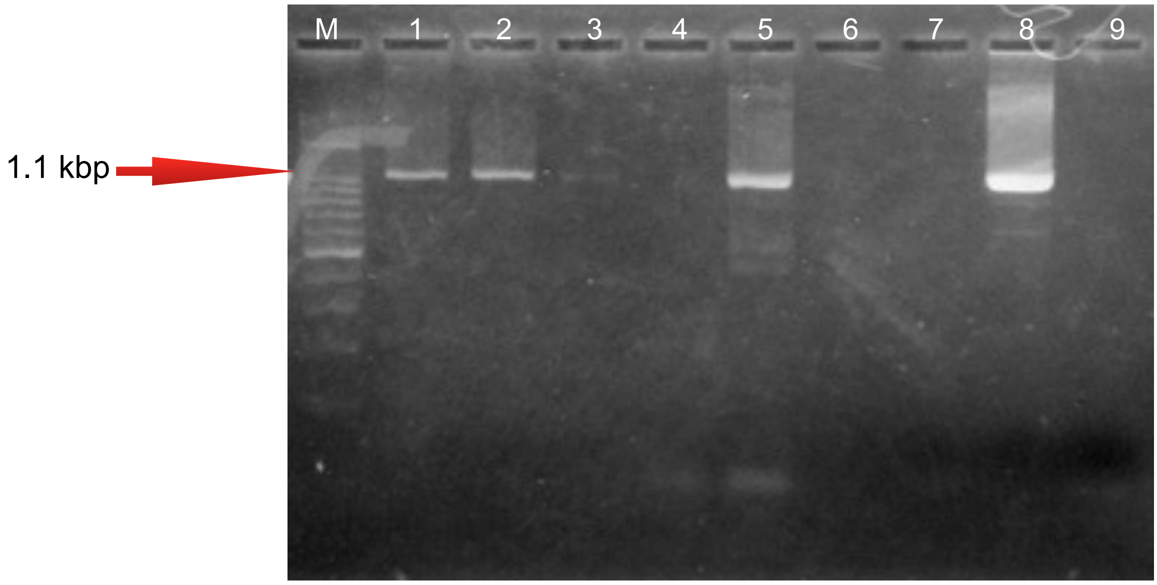

Figure 4: Gel electrophoresis of DNA extracted from banana and abaca leaves. The PCR products were analysed on 2% gel.

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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2. Nelson, S. C. (2004). Banana bunchy top: detailed sign and symptoms. Cooperative Extension Service. College of Tropical Agriculture and Human Resources. University of Hawaii at Manoa. (Retrieved from: http://www.ctahr.hawaii.edu).
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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^{o}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.*

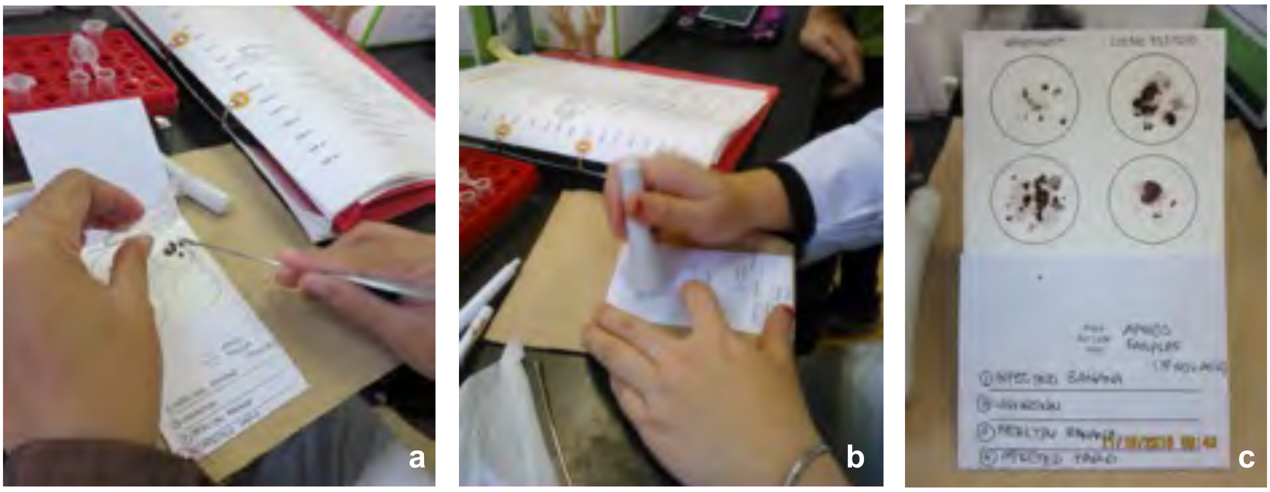

Figure 2: Collection and isolation of DNA from aphids using FTA plant card. (a) placement of aphids; (b) Impregnation of aphids using mortar; (c) the DNA of aphids on FTA plant cards.

Result and discussion

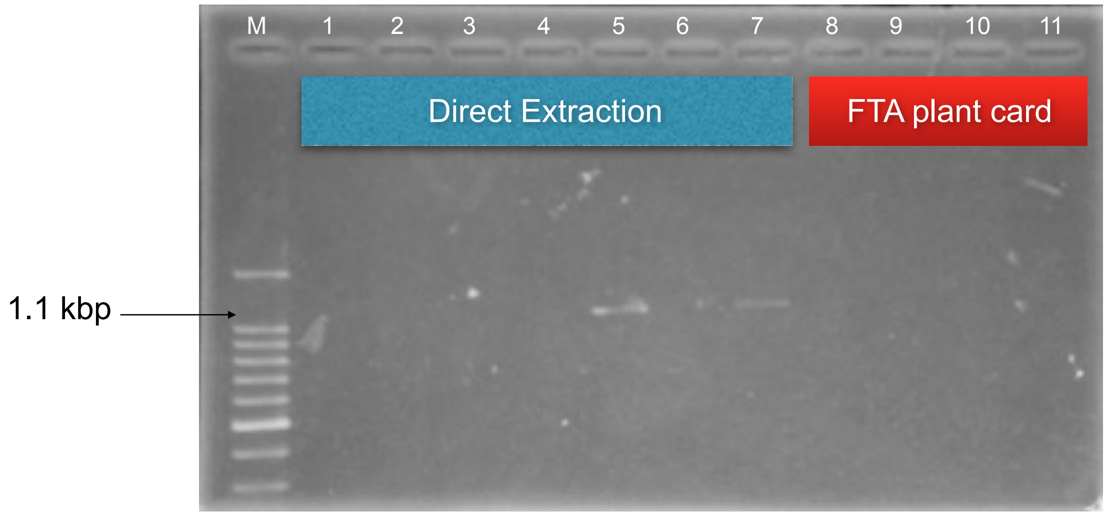

Figure 3: Gel electrophoresis of DNA extracted from banana aphids (direct and FTA plant card). The PCR products were analysed on 2% gel.

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.

References

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2. Crop Knowledge Master: Banana Aphid - *Pentalonia nigronervosa* (coquerel). (Retrieved from: http://www.extento.hawaii.edu).
3. Plantwise Technical Factsheet. Banana Aphid (*Pentalonia nigronervosa)*. (Retrieved from: http://www.plantwise.org).

Appendix 10

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.

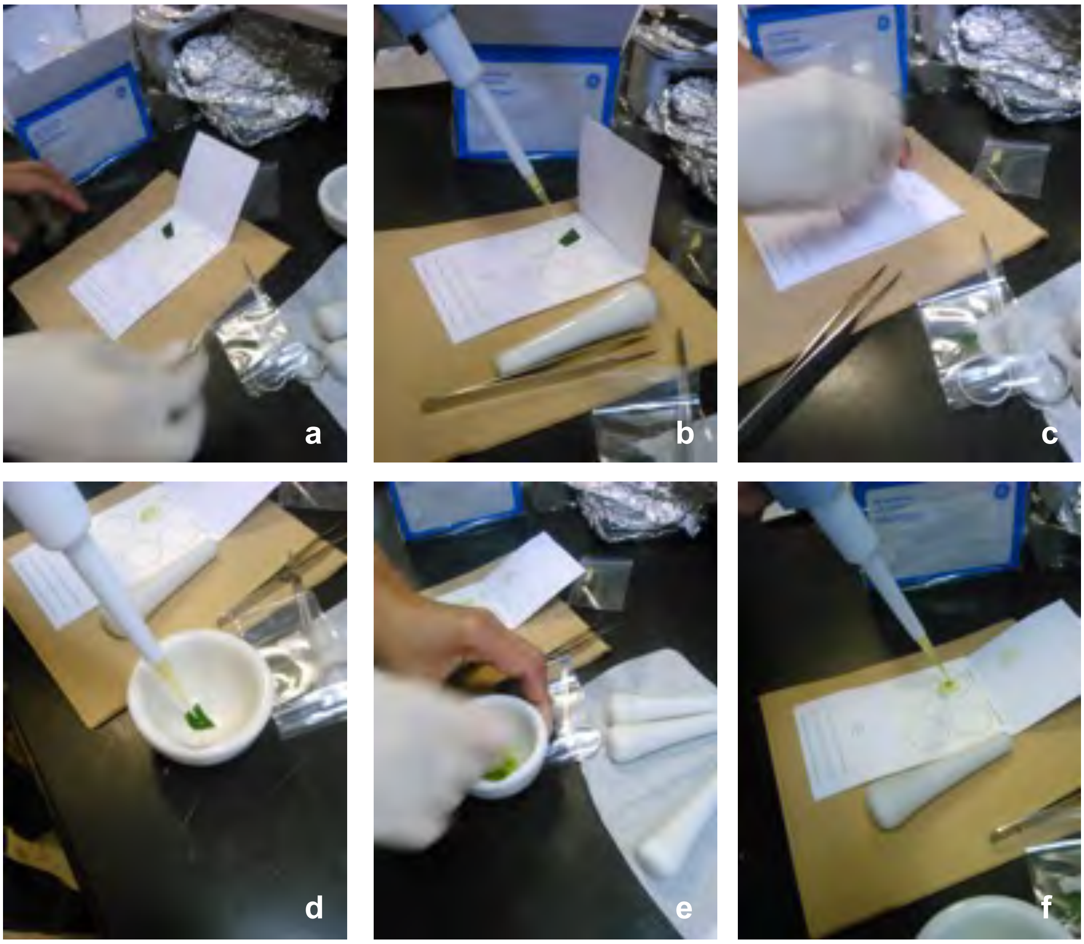

Figure 1: Impregnation samples onto the FTA plant card.
Technique 1 - (a) small size of sample was placed on the membrane; (b) 200 uL of q.s water was put on the sample; (c) sample was crushed using mortar.

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^{o}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

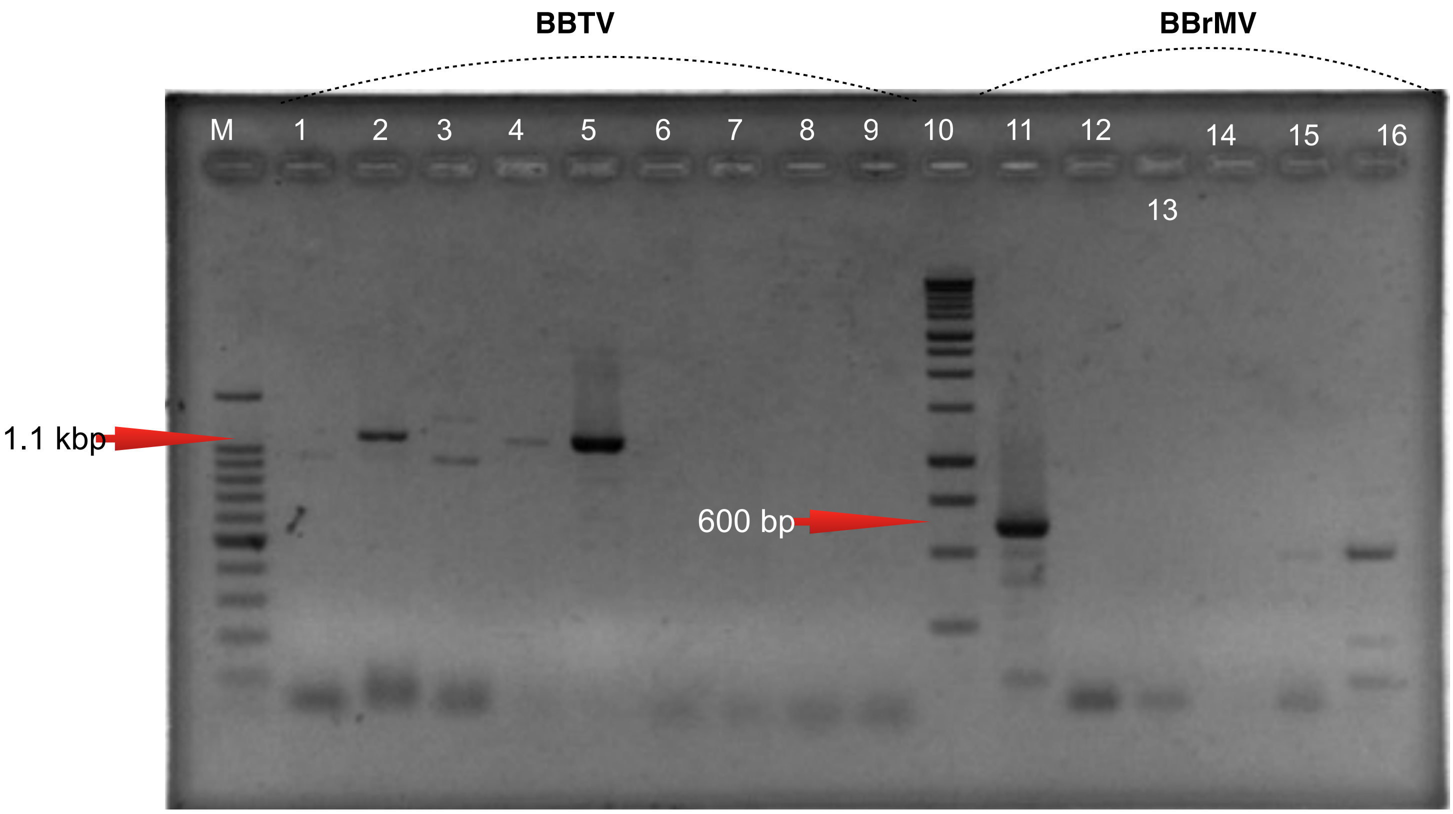

Figure 3: Gel electrophoresis of DNA extracted from banana, abaca and taro leaves for detection of banana bunchy top virus (BBTV) and Banana bract mosaic virus (BBrMV). The PCR products were analysed on 2% gel. Samples were extracted from FTA plant card.

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

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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^{o}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^oC or 20^oC.

e) Ligation (to prepare the recombinant plasmid)

1. All reagent and PCR product was mixed with micropipette and incubated overnight at 4^oC. Longer incubation time will increase the number of transforming. Generally, incubation overnight at 4^oC 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^{o}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^{o}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^{o}C overnight and transferred to 4^{o}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^{o}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^{o}C	10 sec
	step 2	50^{o}C	5 sec
	step 3	60^{o}C	4 min
25 cycle		4^{o}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^{o}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^{o}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^{o}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.

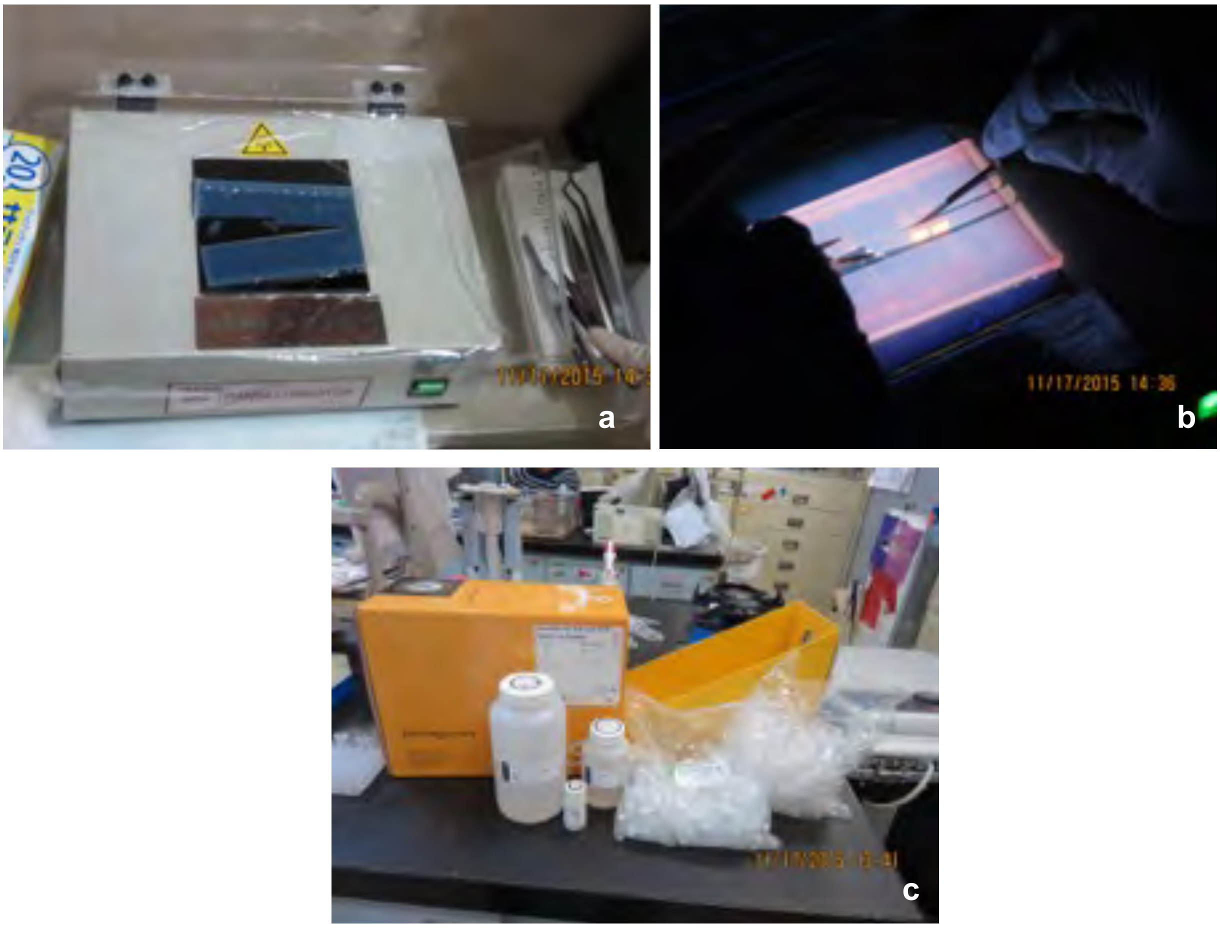


Figure 1: (a) and (b) Gel cutting under UV light; (c) Promega Gel Purification Kit. The gel was then proceed for gel purification protocol.

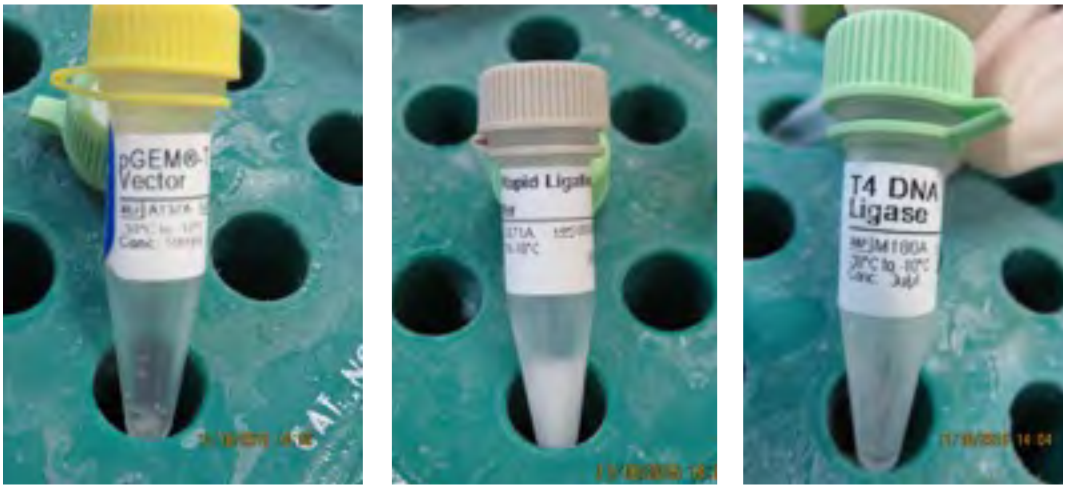


Figure 2: Reagents using for ligation to prepare recombinant plasmid.

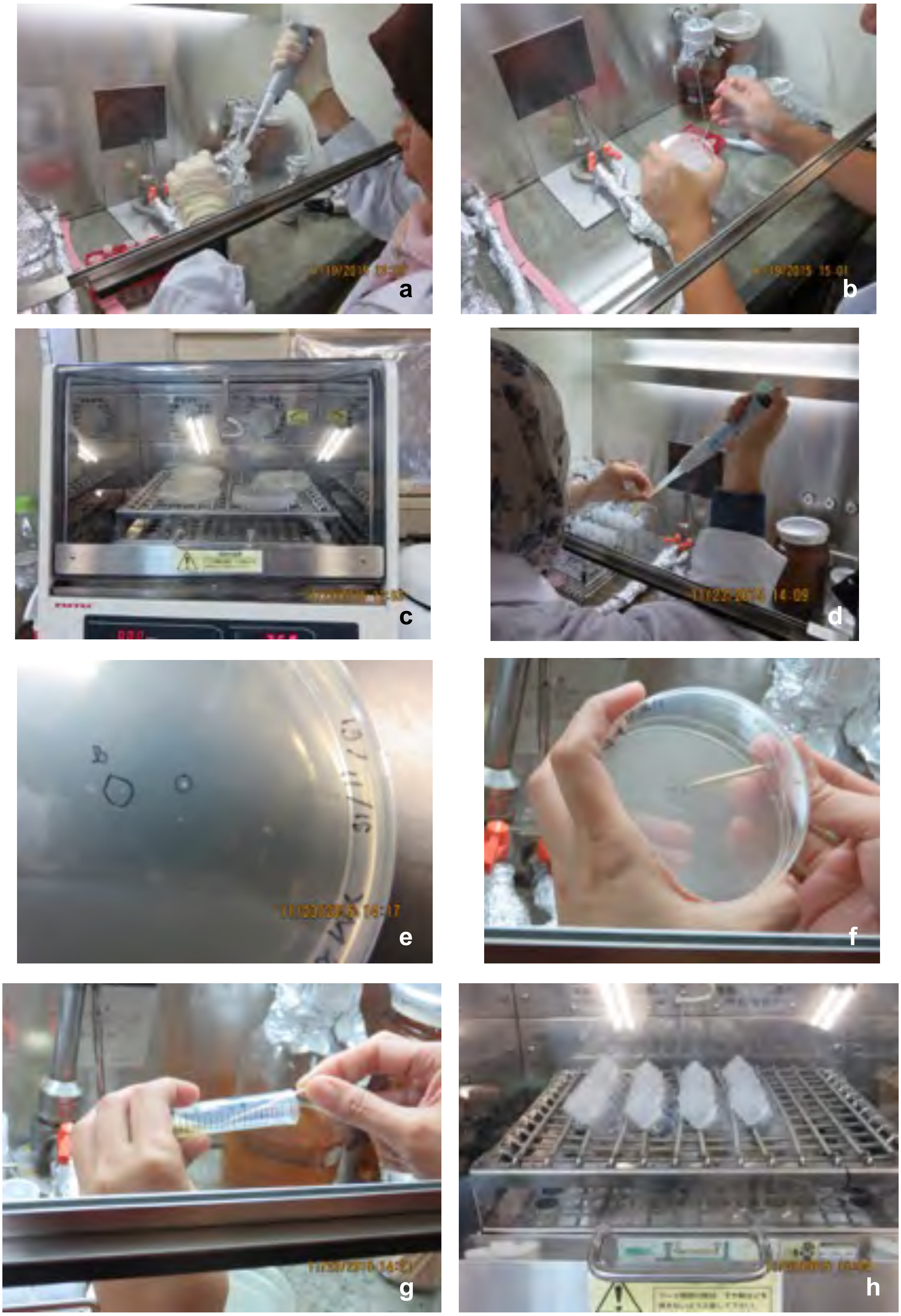


Figure 3: (a) Dispensing of S.O.C medium for production of competent *E. coli*; (b) Culturing competent *E. coli* on LB medium; (c) Incubation of *E. coli* plate in the incubator; (d) Dispensing of TB medium for culturing of E. coli; (e) Blue (without insert) and white (with insert) *E. coli* colony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

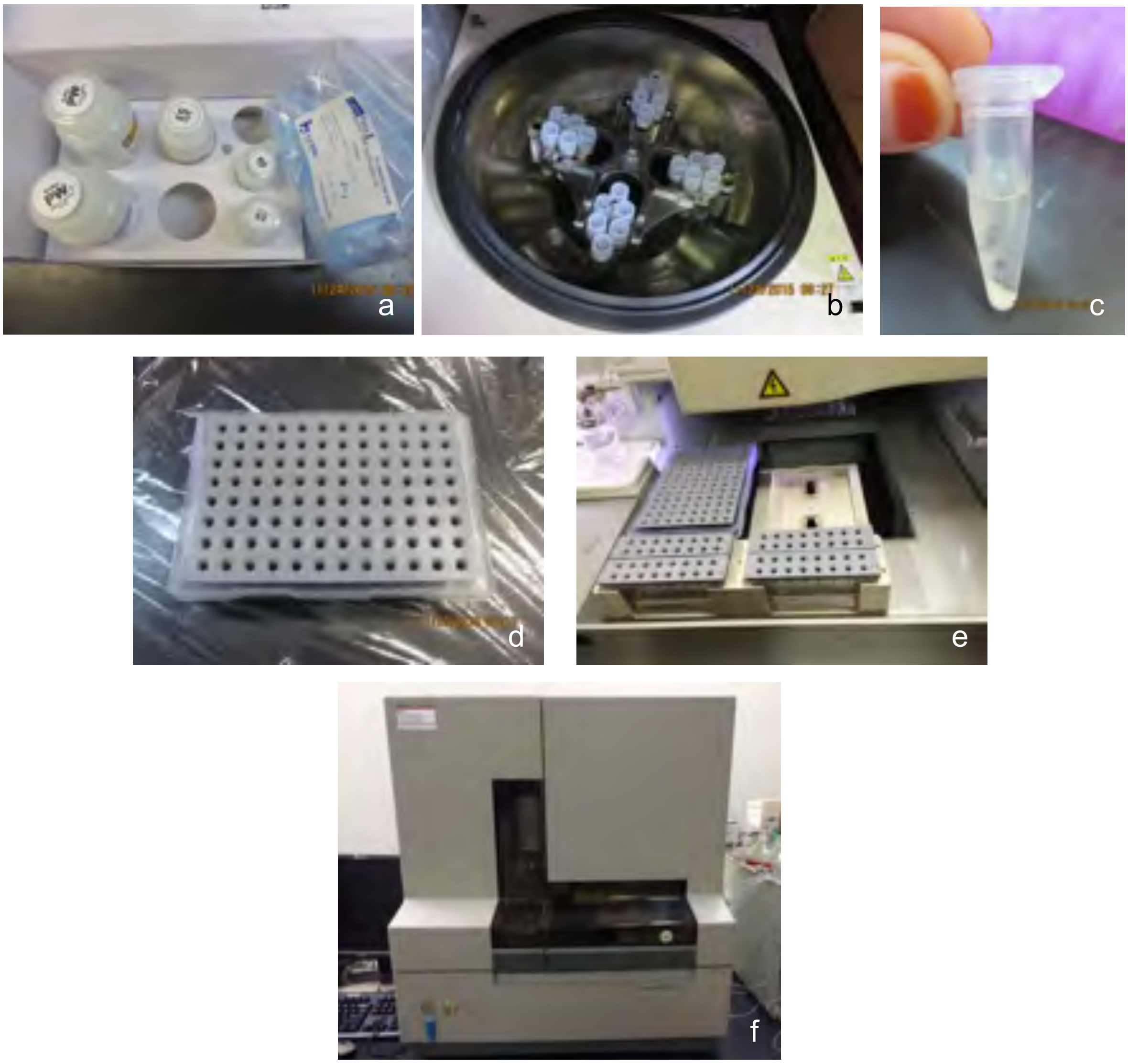


Figure 4: (a) Plasmid Mini Kit for plasmid DNA purification; (b) Plasmid DNA in centrifuge; (c) Precipitation of DNA; (d) Plate using for DNA sequencing; (e) Plate was placed in the sequencer machine; (f) Automate sequencer for DNA analysis.

Results and Discussion

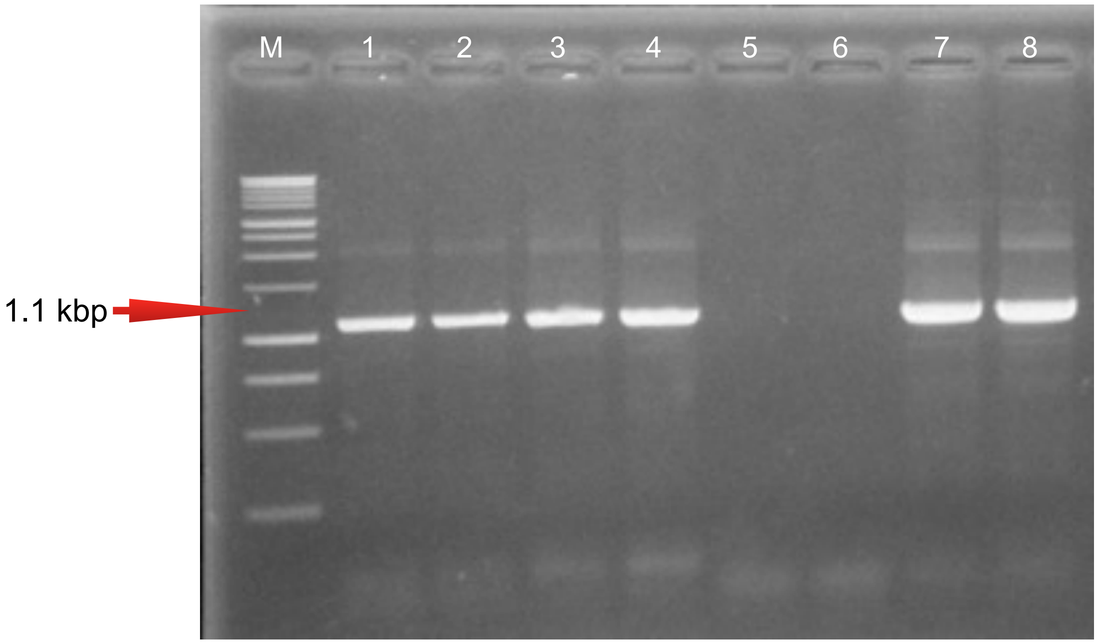


Figure 5: Gel electrophoresis of DNA extracted from banana for gel cutting. The PCR products were analysed on 2% gel.

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).

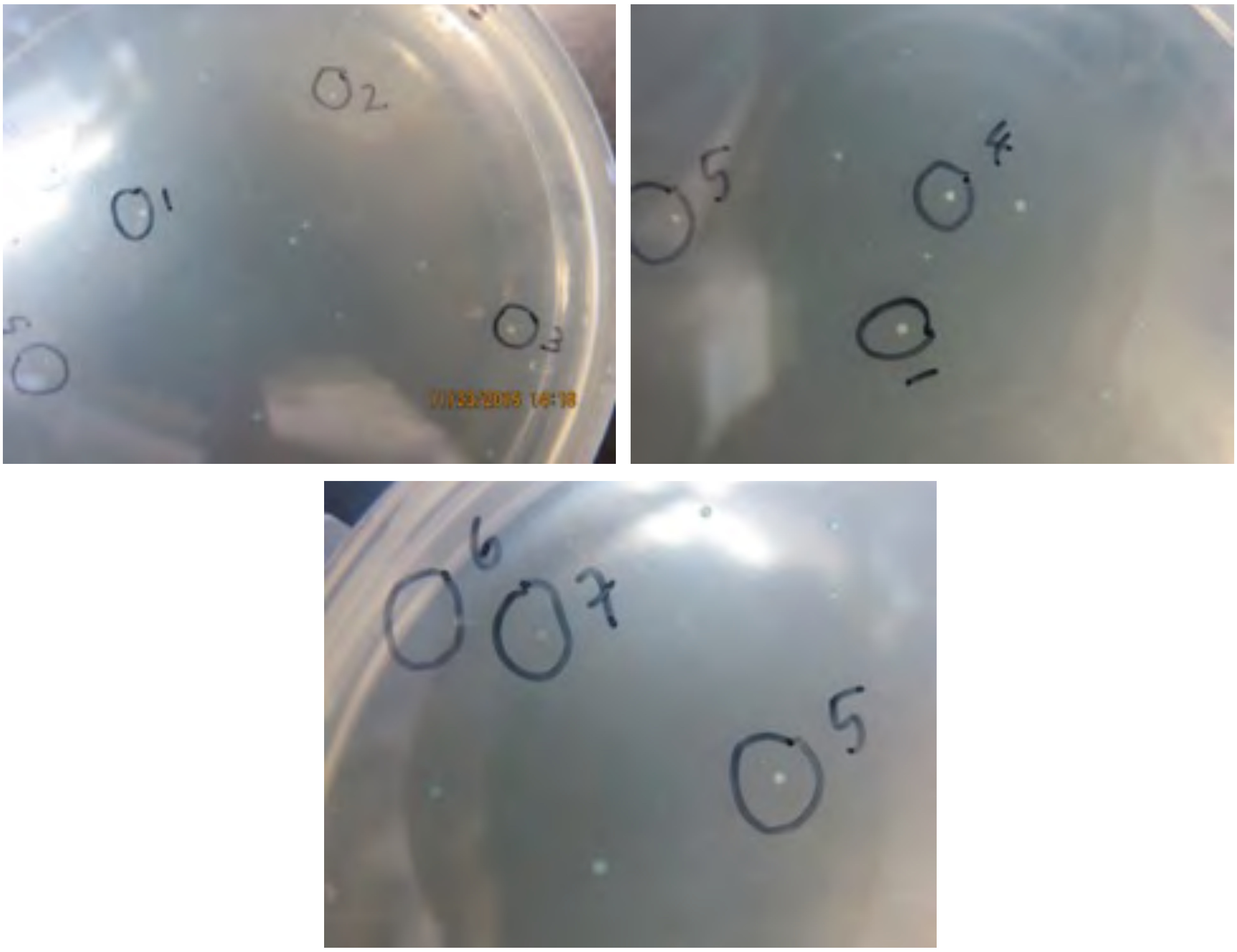


Figure 6: Colony of *E. coli* growth on TB media and was chosen for insert check procedure. White colony labelled with No. 1 - 7 are white colony which indicate to have virus insert. Blue colony (not labelled) have no virus insert.

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.

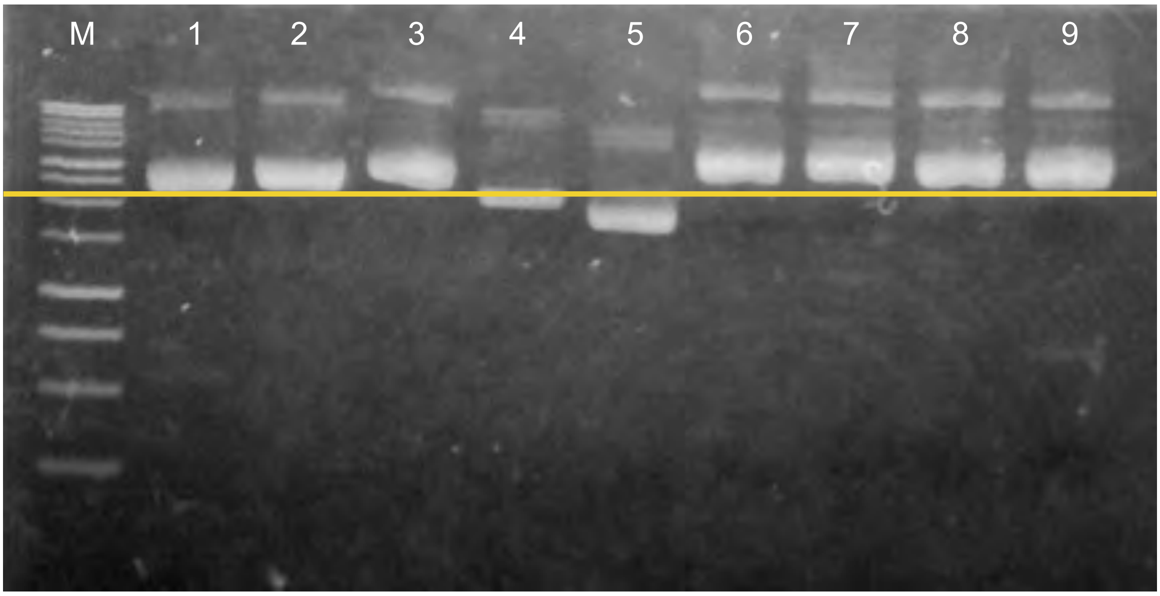


Figure 7: Insert check for plasmid DNA by 1.5% gel electrophoresis. The yellow line indicates the DNA length is 4.1 kbp (3000 bp).

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^{o}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^{o}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^{o}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^{o}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).

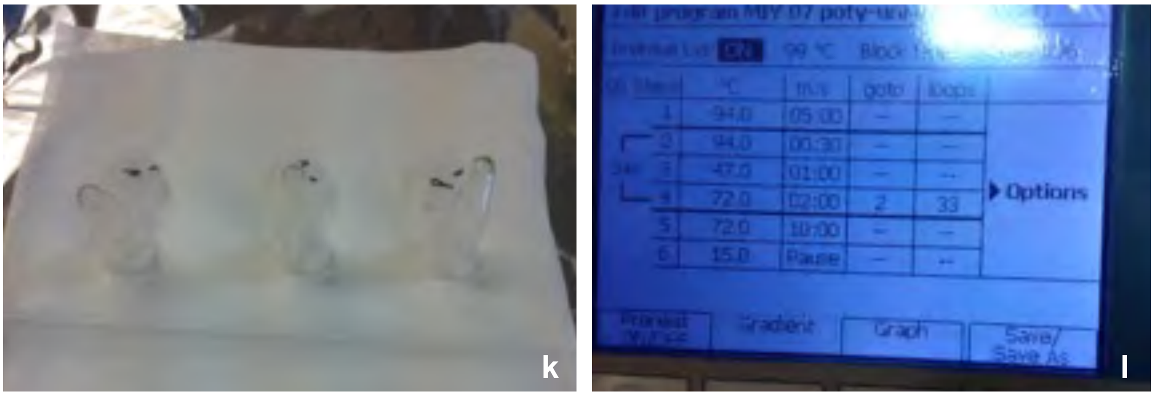


Figure 1: Procedure of RNA extraction. (a) materials needed for RNA extraction; (b) passion fruit infected a month ago with potyvirus; (c) leaf sample was added with liquid nitrogen; (d) sample was mixed with RNA extraction buffer; (e) the sample after additional of phenol chloroform and centrifuged; (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^{o}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^{o}C or 20^{o}C.

g) Ligation (to prepare the recombinant plasmid)

1. All reagent and PCR product was mixed with micropipette and incubated overnight at 4^{o}C. Longer incubation time will increase the number of transforming. Generally, incubation overnight at 4^{o}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^{o}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^{o}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^{o}C overnight and transferred to 4^{o}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^{o}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^{o}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^{o}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^{o}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.

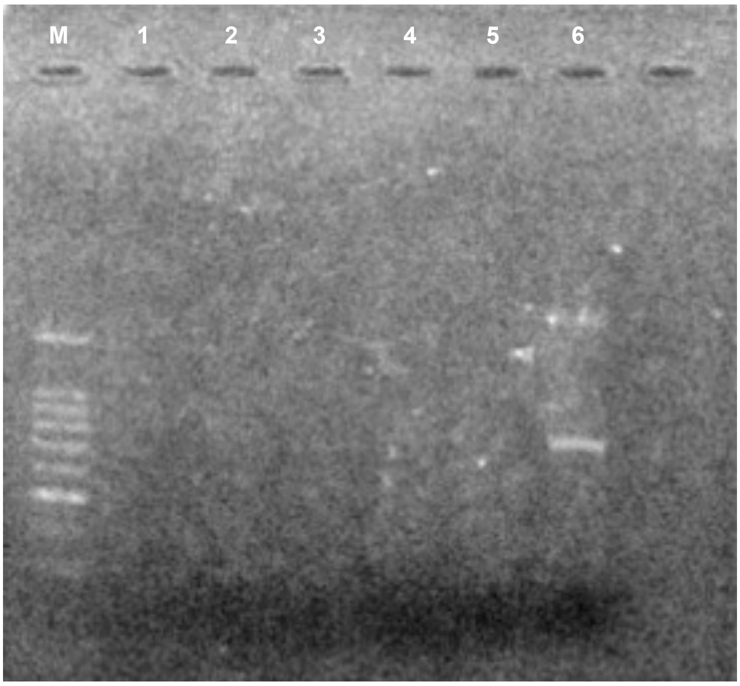


Figure 2: Electrophoresis of RNA from passion fruit infected plant (Potyviruses) on 1.5% agarose gel.

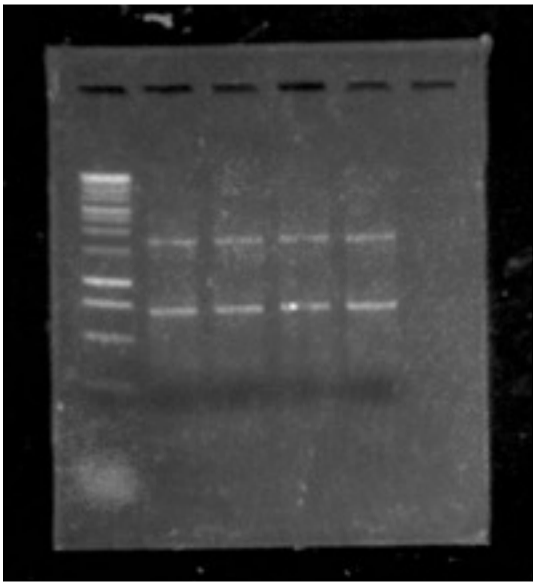


Figure 3: Gel electrophoresis of DNA from passion fruit infected

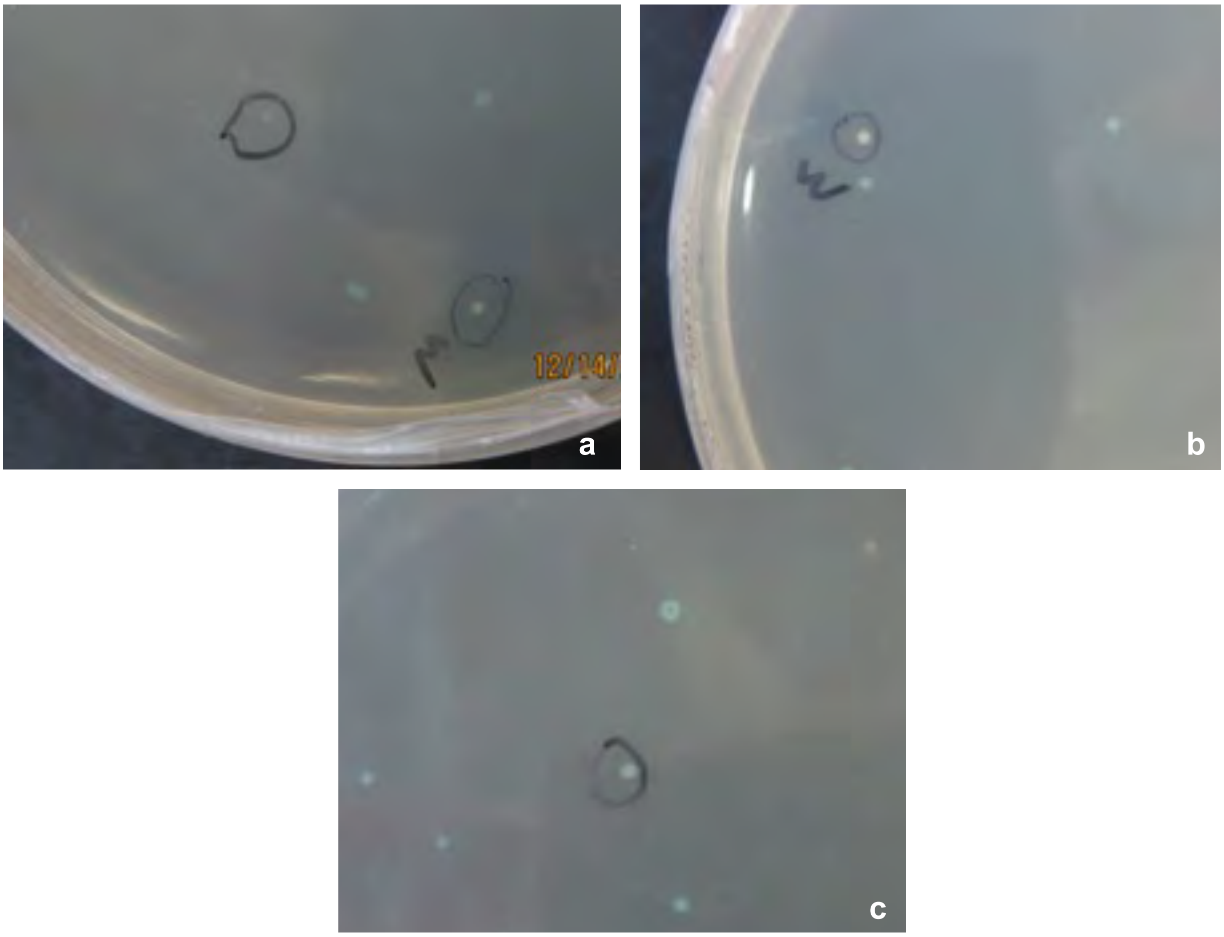


Figure 4: Colony of *E. coli* growth on TB media and was chosen for insert check procedure. (a) and (b) White colony labelled with circular mark are white colony which indicate to have virus insert; (c) Blue colony (not labelled) have no virus insert.

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.

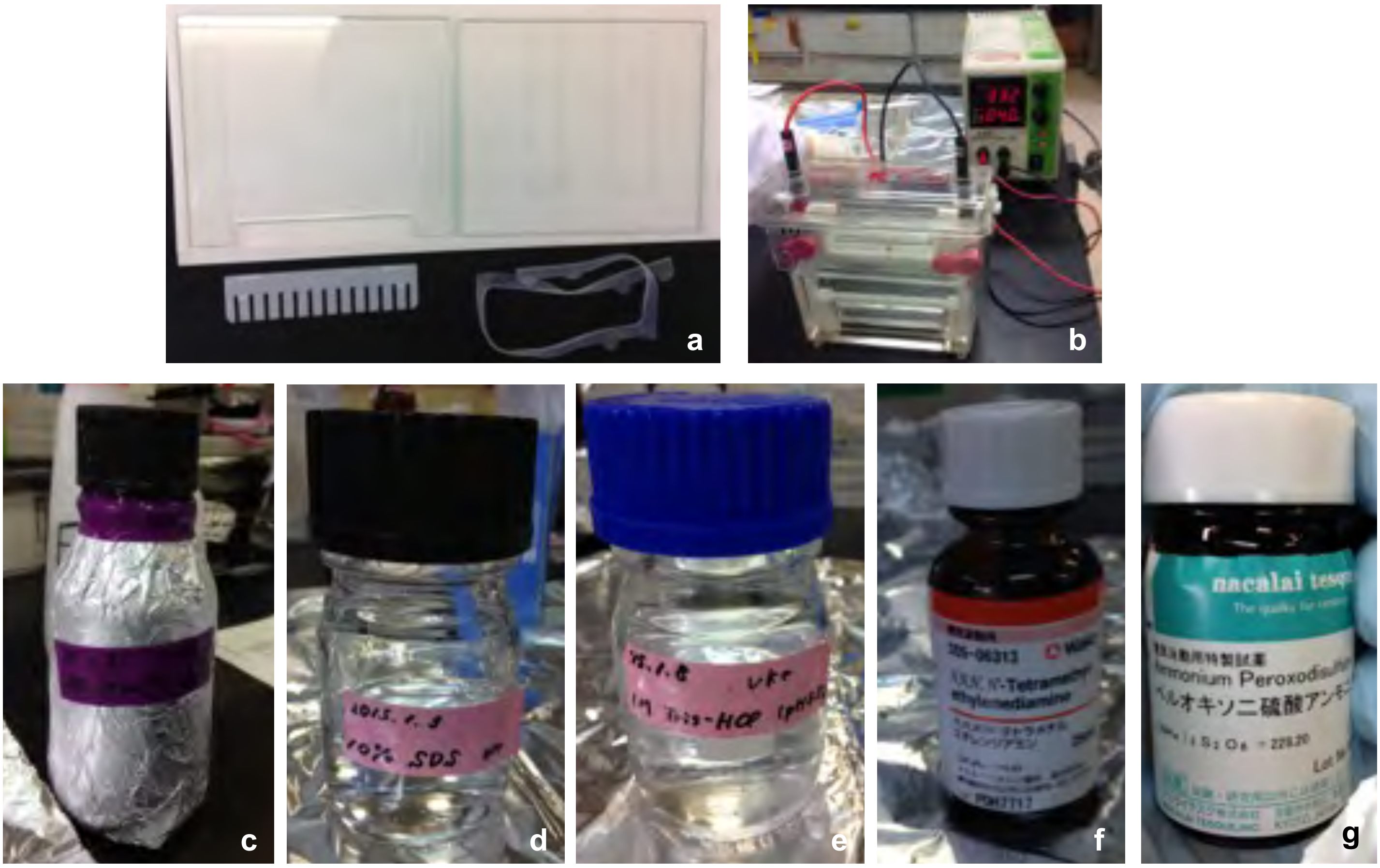
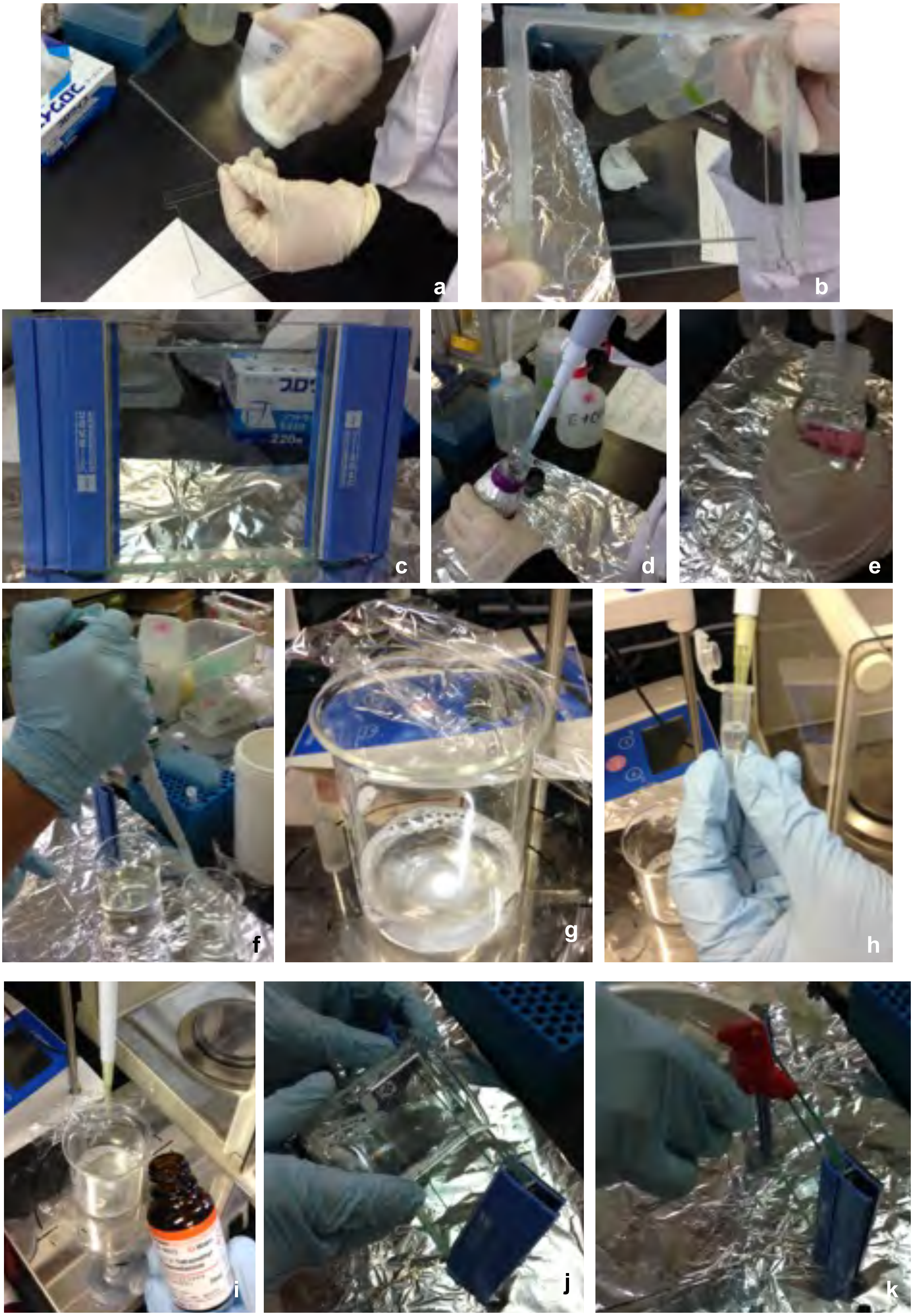


Figure 1: Equipments and reagents used for gel preparation. (a) Equipments to make gel (square glasses, comb and spacer); (b) gel electrophoresis & electric supply; (c) 30% acrylamide solution; (d) 10% SDS; (e) 1 M Tris-HCl (pH 8.8); (f) TEMED; (g) Ammonium peroxodisulfate.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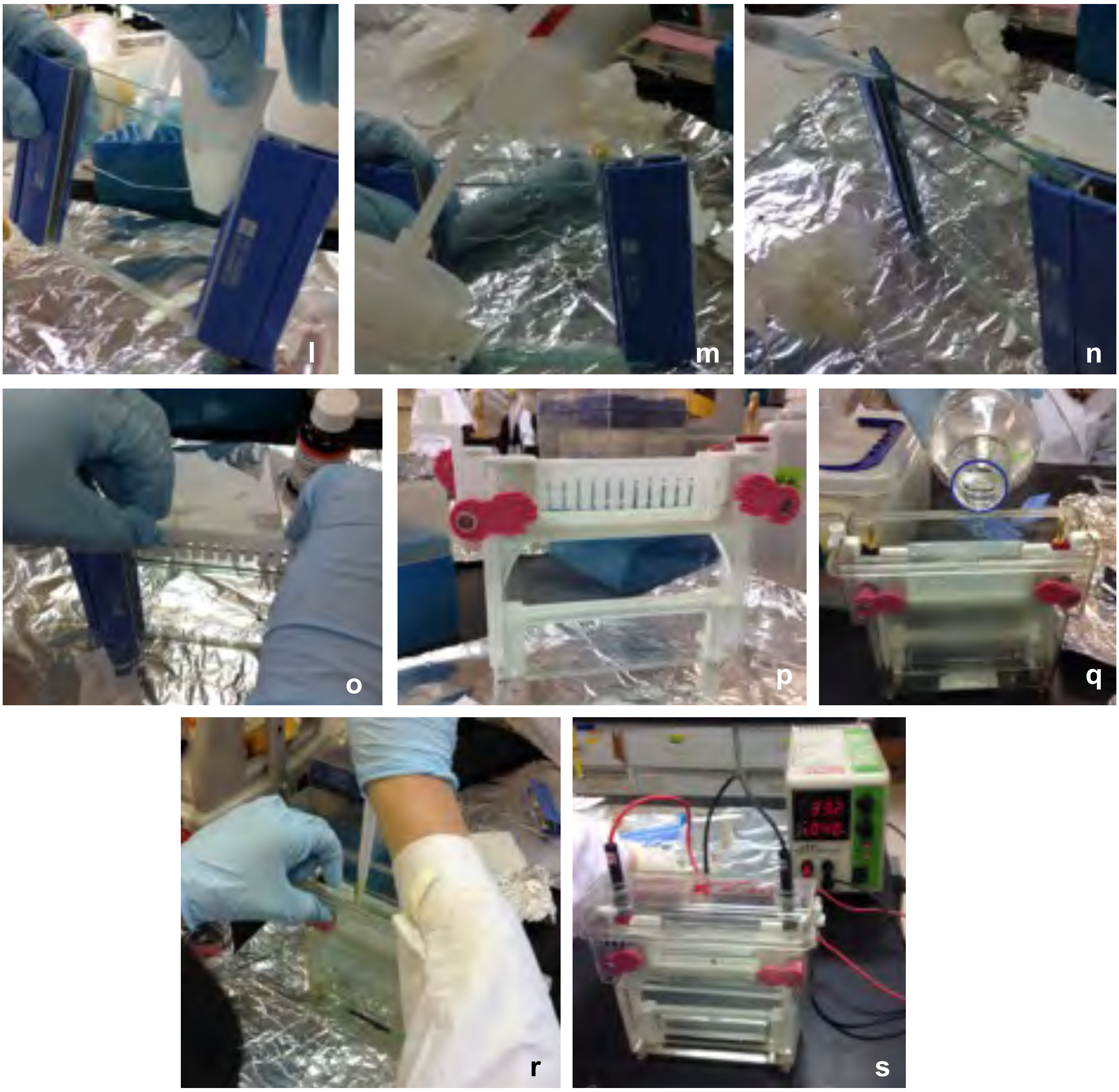

Figure 2: Procedures to make the gel. (a) Disinfected with 70% alcohol; (b) and (c) assembled all the equipments; (d) 30% acrylamide gel added into the beaker; (e) additional of 10% SDS; (f) The reagents were mixed with distilled water; (g) The solution was stirred; (h) Additional of 10% AP; (i) Additional 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^{o}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.

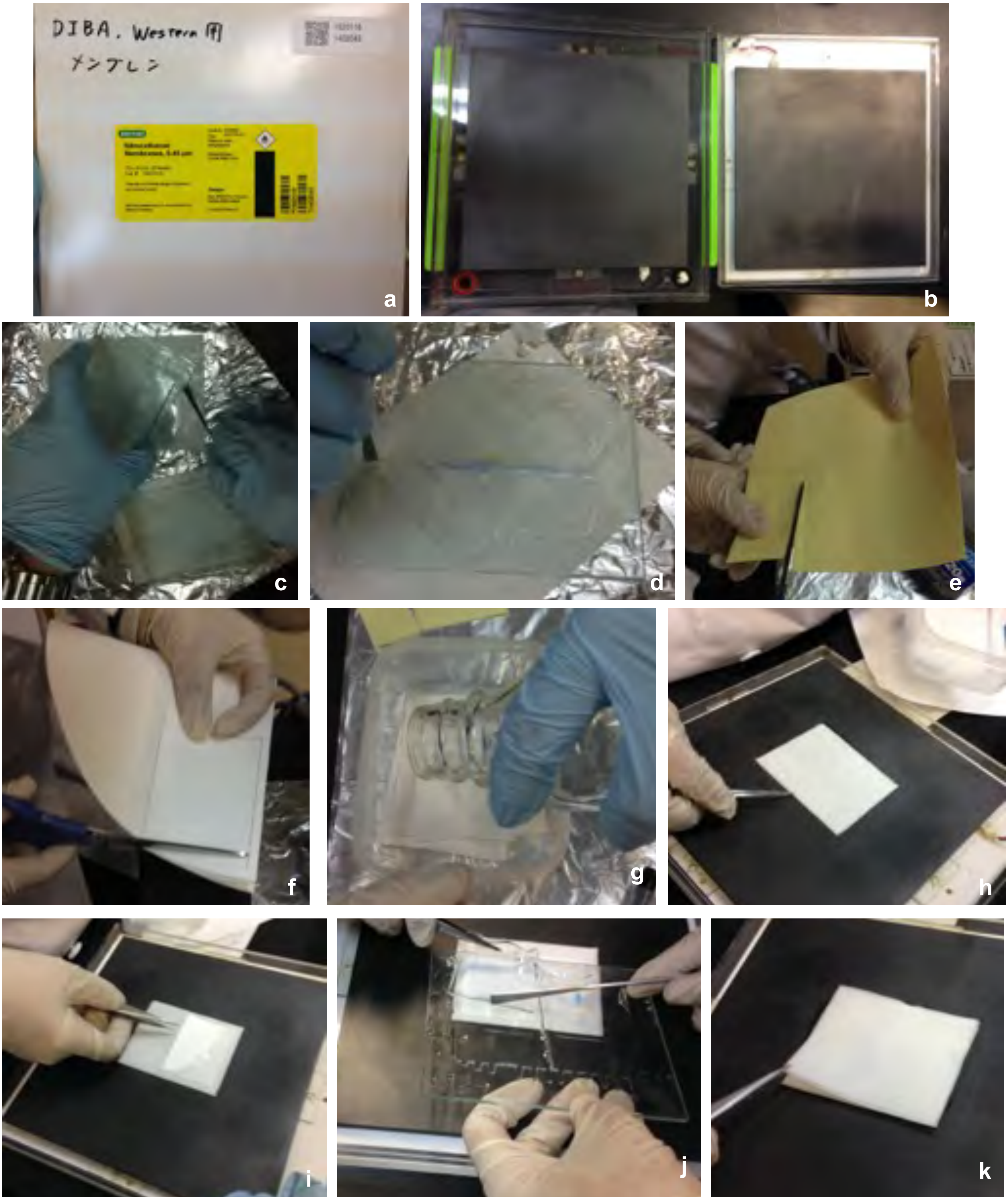
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Figure 3: Western Blot procedure. (a) Membrane for Western Blot test; (b) Western Blot divide; (c) Gel was taken out from squared glasses; (d) Gel was cut to separate the protein which only content protein bands; (e) Membrane was cut according to gel 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

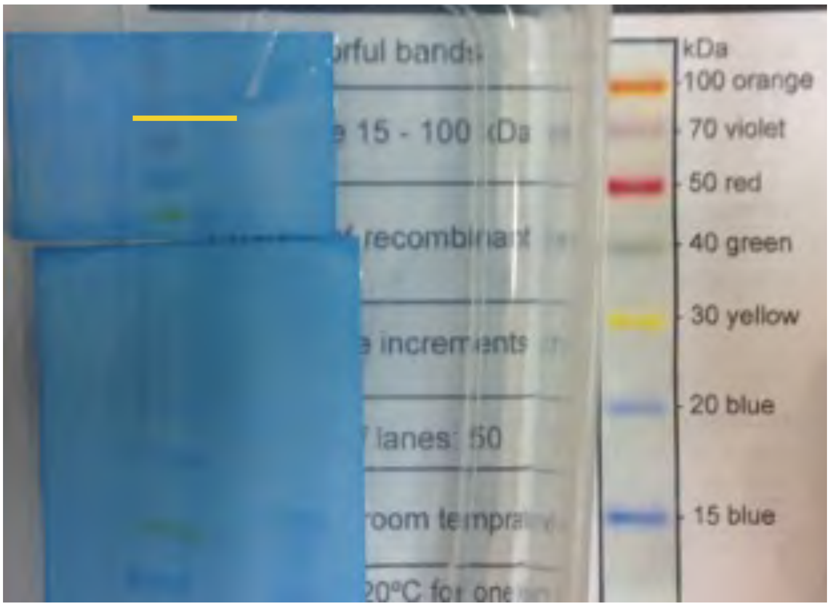

Figure 4: The band was appeared on the membrane after Western Blotting procedure.

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)

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^oC 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^oC) 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^oC overnight or 80^oC for 1 hour.
18. After storage overnight, the sample was centrifuged in cold condition (0^oC) 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.

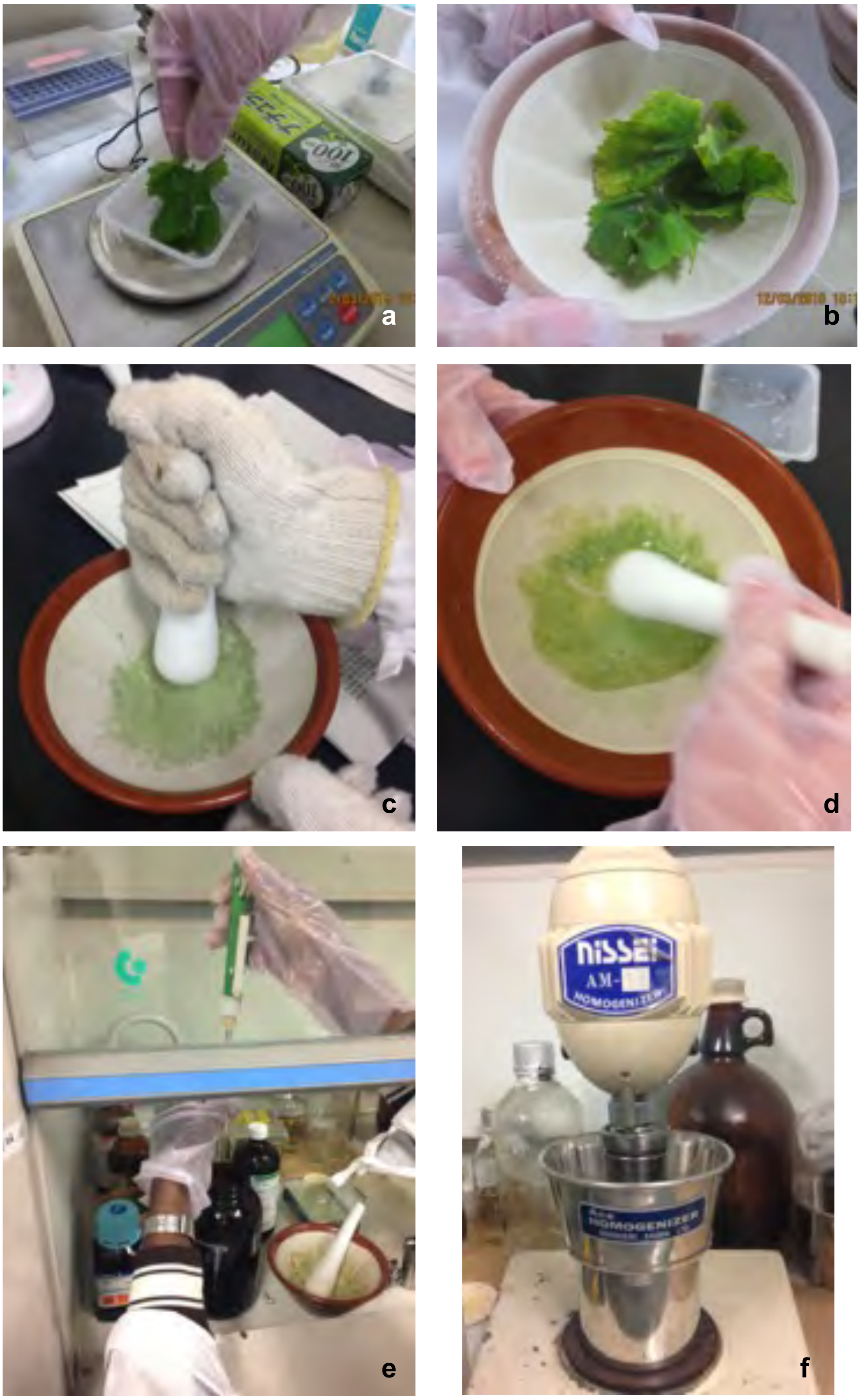

Figure 1: Procedure of dsRNA extraction. (a) weigh of infected leaf sample; (b) leaf sample was placed into cold pastel; (c) sample was grounded into powder; (d) sample was homogenised with STE buffer; (e) phenol, chloroform and pentanol was added into remaining sample; (f) mixture was homogenised using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

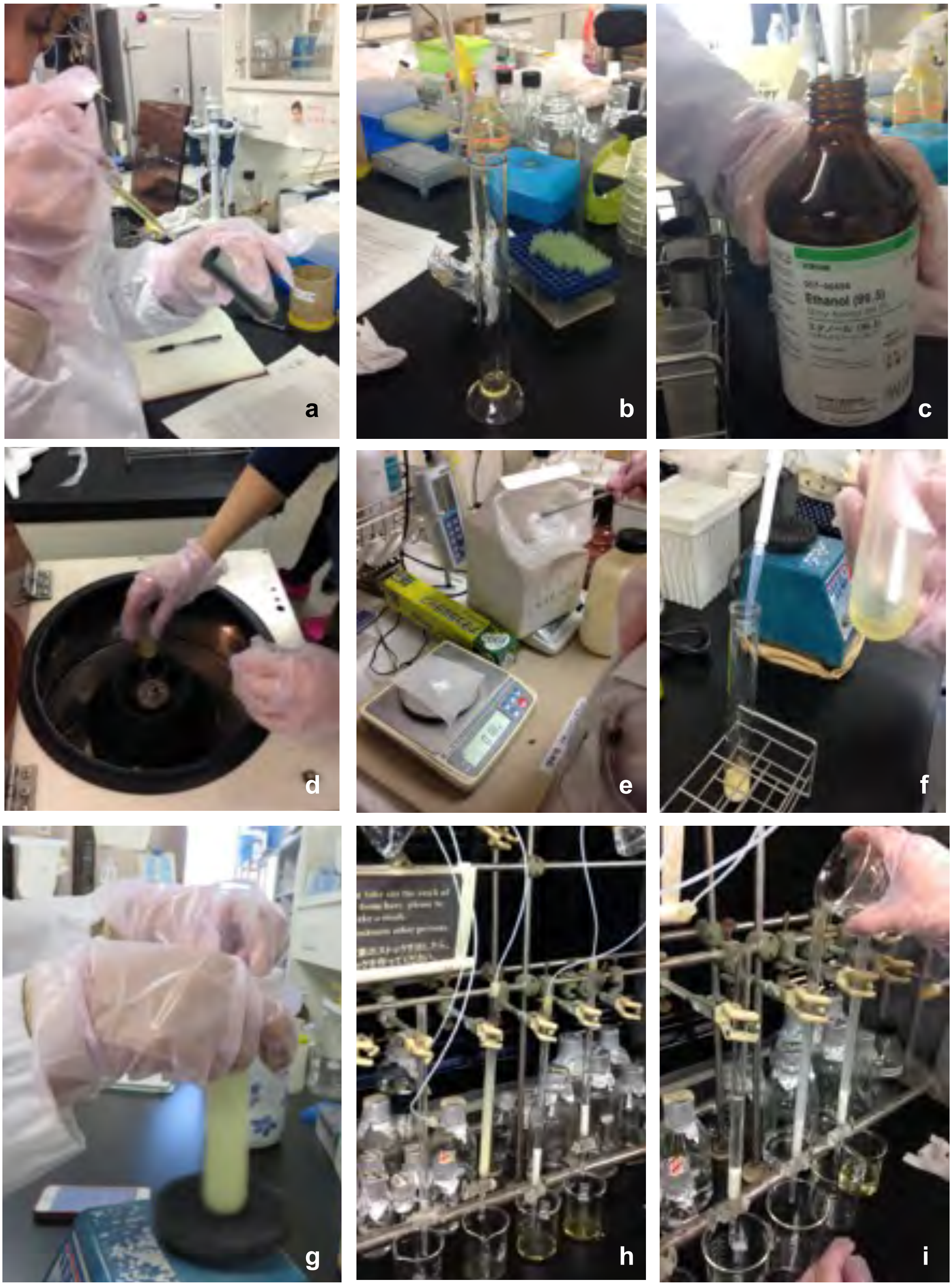

Figure 2: Procedure of dsRNA extraction. (a) supernatant was transferred; (b) volume supernatant was measured with measuring cylinder; (c) ethanol was added into the supernatant; (d) sample was mixed and centrifuged; (e) CF-11 cellulose powder was weigh; (f) supernatant was added into cellulose powder; (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.

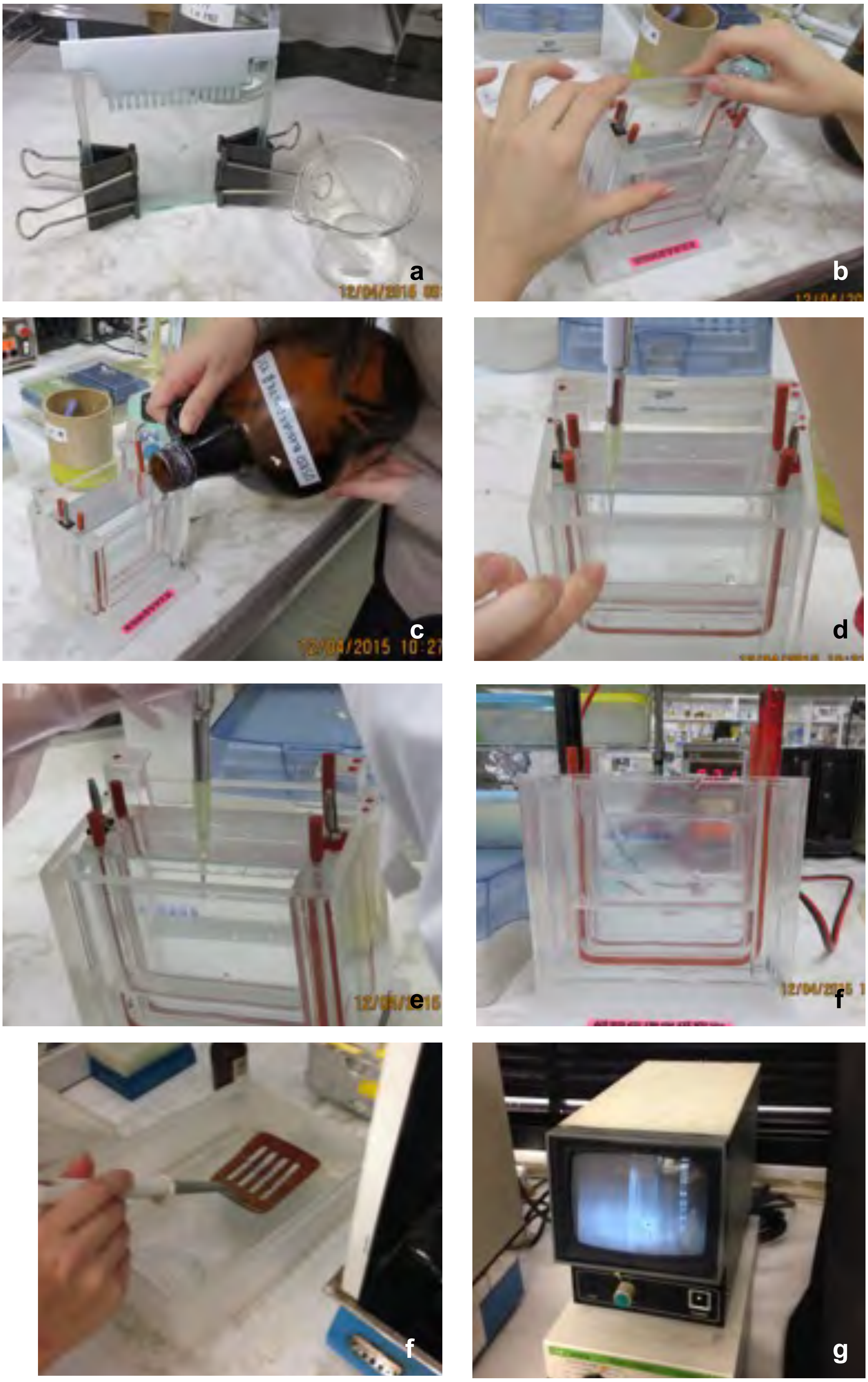

Figure 4: Procedure of dsRNA extraction. (a) polyacrylamide gel setting; (b) after gel was solidified, the gel was placed inside the main case; (c) 1X TBE buffer was added into the gel case; (d) 1 kbp marker was loaded into the first well; (e) all samples were loaded into each well; (e) gel was ran for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

Results and Discussion

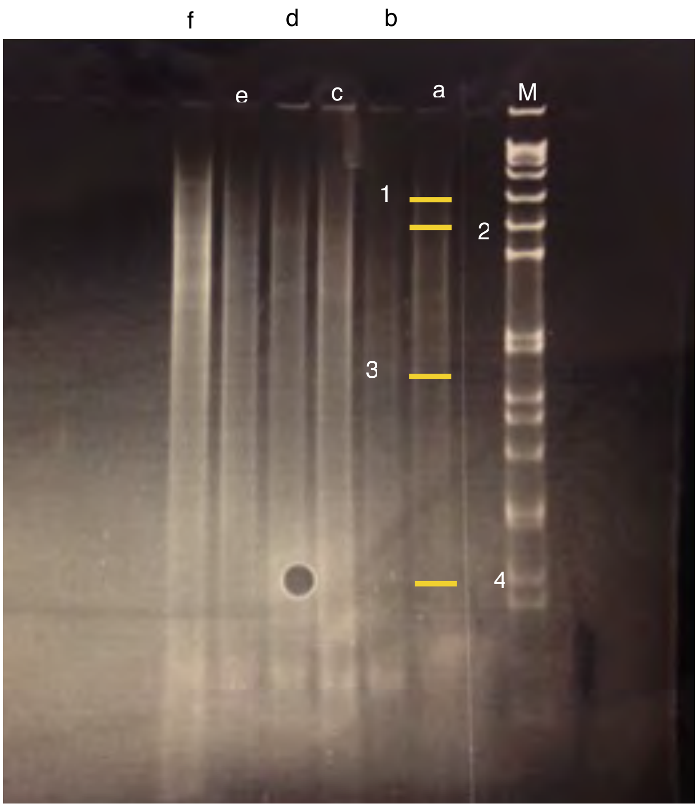

Figure 5: Electrophoresis of dsRNA from Cucurbit infected plant (Cucumber mosaic virus - CMV).

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.

References

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2. Morris, T. J. and Dodds, J. A. (1979). Isolation and analysis of double-stranded RNA from virus-infected plant and fungal tissue. Phytopathology, 69: 854 - 858.
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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.

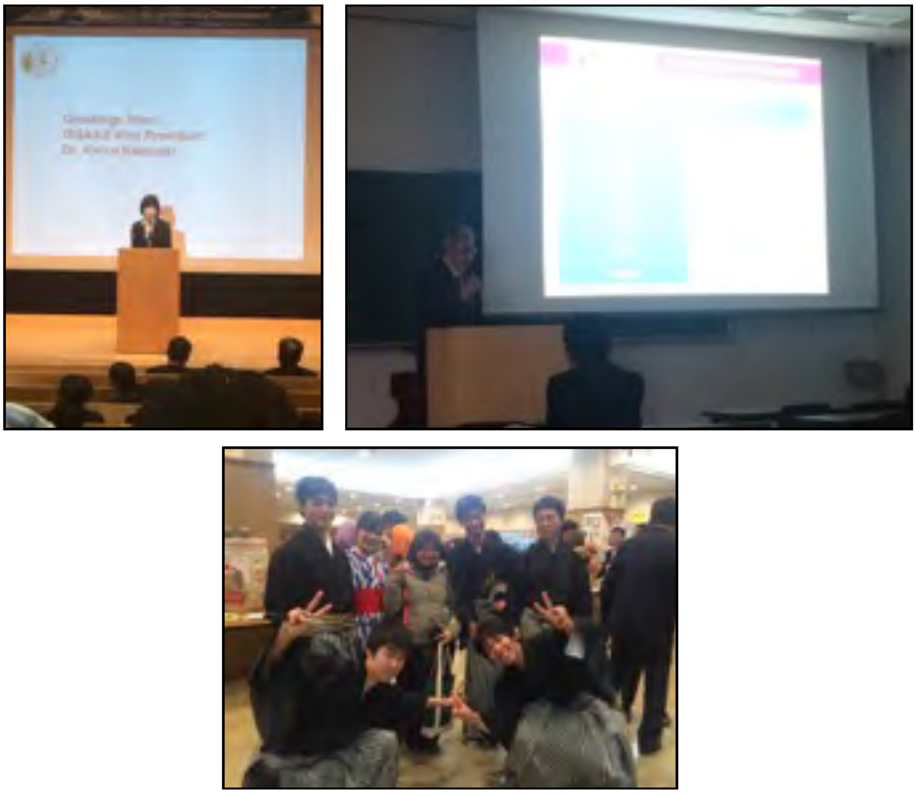
Appendix 16

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