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INCIDENCE AND DISTRIBUTION OF THREE VIRUSES INFECTING PLANTS IN THE FAMILY CURCUBITACEAE IN COAST AND DAR ES SALAAM REGIONS OF TANZANIA

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ABSTRACT

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The objective of this thesis is to study viruses infecting plants in the family *curcubitaceae* in Coast and Dar es Salaam regions in Tanzania. The focus is on watermelon, cucumber and pumpkin farms and to study the incidence and distribution of three different viruses called *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus* (WMV).

Samples were collected from farms on Coast and Dar es Salaam Regions, Tanzania. On the farms the farmers were asked questions about their experience about the viruses and diseases on the farms. The samples were tested to laboratory using ELISA-method and the results were read visually and by using ELISA plate reader.

26% of the collected samples were infected either with CMV, ZYMV or WMV. Some of the plants were infected with two of the viruses, one plant was infected by all three viruses. Only on 10% of the farms there were no symptomatic plants found and samples were tested negative, from 90% of the farms symptomatic plants were found and part of the samples were tested positive. The highest percentage of infected plants on one farm was 80%.

Keywords: Zucchini yellow mosaic virus, Cucumber mosaic virus, Watermelon mosaic virus, ELISA method, Family curcubitaceae

PREFACE

The thesis was made in Dar es Salaam, Tanzania on February and March of 2016. The study was made for Mikocheni Agricultural Research Institute (MARI). The laboratory work was done on laboratory of MARI and the field work was done on the Coast and Dar es Salaam regions in Tanzania.

Study was done for the project lead by Doctor Deusdedith Mbanzibwa. The laboratory work was done with the assistance of the members of the MARI. The tutoring teacher in Finland has been Joni Kosamo. Also Finnish teacher Pirjo Partanen and English teacher Marjo Heikkinen have been tutoring this thesis.

Laboratory work in Tanzania has been quite an experience compared to working in Finland. There have been continuous problems during the work due to lack of water, electricity and equipment. Any of those is not an excuse to stop working. Creativity is something that local people have learnt to use and that has been very instructive for me. Field work as well has been an invaluable experience.

Special thanks for the farmers and local people on the field. Their help and assistance have been very important and valuable, the work could not have been done without them.

CONTENTS

1 INTRODUCTION

This chapter explain the background of the study. Tanzania and its agriculture is introduced shortly in first subchapter. The second subchapter is about mosaic viruses and the third one is about ELISA method which have been used in this study.

1.1 Tanzania

Tanzania is a country in the continent of Africa located in east coast bordered by Indian Ocean. Specific location is shown on the *FIGURE 1*. It is also bordered by Uganda, Kenya, Burundi, Rwanda, Congo, Zambia, Malawi and Mozambique. (1)

FIGURE 1 A map of Tanzania (2)

1.1.1 United Republic of Tanzania

The country called Tanzania (Long form: The United Republic of Tanzania) became independent from the United Kingdom on 9 December 1961. Nowadays the population of Tanzania is approximately 48 million people. Tanzania covers 947 000 km^2 of land and has density of 49 people / km^2. (2) The capital city of

Tanzania is Dodoma, although Dar es Salaam is the largest city of the country. (3)

The official languages are Swahili and English although over 100 languages are spoken around the country. Major religions are Christianity, Islam and various traditional African religions. Currency is Tanzanian shilling. The president of Tanzania is John Magufuli (2016). (4)

Tanzania has a great variation of altitude having the beaches, the Great Plains and the mountains. Despite the richness of the wildlife and landscapes Tanzania is one of the poorest countries in the world having serious environmental problems (5). One third of the population is living below the poverty line (2012). Inequality between rural and urban population has increased and about 12 million people in Tanzania are living in poverty. Challenges are to improve the business environment, build skilled and healthy workforce and increase agricultural productivity. (6)

1.1.2 Agriculture

Tanzania has great opportunities for agriculture-led economic growth having everything that is needed; rich land, water resources and motivated farmers. The climate gives possibilities for a variety of crops. (7) Local products are sold in markets around the country (*FIGURE 2*).

FIGURE 2 Watermelons in local market (Photo by Maria Sydänmetsä)

An average Tanzanian farmer is a smallholder. Only a minority cultivates more than two hectares. People may even have access to more land but because of lack of agricultural tools they are not able to exploit that. Farmers use tools that are very basic hand held tools and most of the farmers do not use animals for ploughing. (5)

In terms of crops maize and cassava dominates most of the Tanzania. Rice is grown in some part of the country. Because Tanzania has such a variety of climatic and geographical zones farmers have opportunity to grow big variety of vegetables and fruits. Coffee is a big export crop, as well as cotton, cashew nuts and tobacco. Tanzania is amazingly self-sufficient on food production. Over 90 % of food is home grown. And yet farmers suffer from poverty. In rural areas only one third have access to safe water and malnutrition is common amongst the farmers. (5)

1.1.3 *Cucurbitaceae* plant family –farming in Tanzania

A gourd family called *cucurbitaceae* consist of many different species. Cultivated species of this family are easily recognizable. Cucurbits can be found native in many countries over the world, especially in tropics. Cucumbers can be cultivated everywhere in warm temperature. Edible species of cucurbits are cucumbers, pumpkins, melons and squashes. (8)

Cucumber (*Cucumis sativus*) is the most well-known vegetable plant and it is cultivated all over the world, because it can also be cultivated in green houses. The cucumber crop is best grown on warm season regions and it is very sensitive to cold. (9)

Zucchini (*Curcupita pepo*), also called summer squash, is cultivated all over the world. Squash is a warm season plant and it grows best in hot climate conditions. It can't tolerate cold. (10)

Watermelon (*Citrullus lanatus*) is grown in tropical and sub-tropical areas of the world. It is warm long-season crop. Watermelon is cultivated because of its juicy fruit. The farmers can make good profit of it if the proper cultivation method and proper farm management are allowed. Watermelons cannot tolerate cold, any frost can severely damage all the crop. Watermelon crop demands sunshine and dry weather, cool nights and warm days are ideal for the fruit. (11)

Pumpkins (*Curcupita pepo*) are also warm-season vegetable and are very tender to the cold. Depending on the variety pumpkin can also be called squash, which may cause some confusions. (12)

1.2 Viruses causing mosaic symptoms

Mosaic viruses are plant viruses affecting in many different plants (vegetables and flowers) causing big economical losses worldwide on the crop production.

Even though symptoms and severity vary with the type of plant, conditions and the strain of virus, the most of the symptoms are similar and usually very visible.

The viruses cause yellow mottling on leaves, distortion and stunted growth. Identification can be conform by ELISA using specific antibodies. (13)

As soon as the plant has been infected, there is no cure or treatment. The most effective method is to purchase plant material that is virus-resistant. (14) Because mosaic viruses are usually transmitted by aphid, using insecticides is not practical. The best way to avoid these viruses is to obtain genetically resistant crops. (15)

This study focuses on three of the mosaic viruses called *Cucumber mosaic virus*, *Zucchini yellow mosaic virus* and *Watermelon mosaic virus*. All of them are causing diseases on the *cucurbitaceae* plant family.

1.2.1 *Cucumber mosaic virus* (CMV)

CMV has been described already in 1916 and it occurs worldwide affecting crops causing big economical losses in tropic, sub-tropic and temperate areas in the world. Crop losses vary every year, because amount of viruses depends upon the aphid population. Cool and wet fall or spring numbers of aphids decrease, but if the spring or fall is warm and dry numbers of aphid populations increase rapidly and virus spreads quickly into crops. Usually crop losses are 10-20 % but in the worst cases it can be even 100 %. (15)

Cucumber mosaic virus CMV has one of the broadest host ranges, virus affects in many different plants and even flowers, for example peppers, spinaches, lettuces, celeries, beans and tomatoes (16). It can infect almost 1200 plant species (15). Visible symptoms are yellow mottling on leaves, distortion and stunted growth. Yellow mottling on leaves and mosaic is shown in picture *FIGURE 3* (16).

FIGURE 3 Symptoms of CMV (17)

1.2.2 *Zucchini yellow mosaic virus* (ZYMV)

ZYMV has been recognized in 1981 although symptoms were described already in 1973. Nowadays it occurs in five continents causing devastating epidemics worldwide (18). ZYMV causes the economically most important diseases of cucurbit crops (19) and with *cucumber mosaic viruses* and *watermelon mosaic viruses* it can be major limiting factor for the cucurbit production (20). Before fruit set infected crops can loss be even 95% its yield (13). Host range is quite wide and ZYMV has been found at least in winter squash, cucumber, pumpkin, watermelon and melon (20).

ZYMV is a potyvirus with single strand of RNA. ZYMV is spread by aphid species. Being a non-persistently aphid-transmitted virus ZYMV is difficult to control with reflective mulches and insecticides. The best result would obtain with resistant cultivars. (18)

Symptoms occurring of ZYMV infections are visible. Plants are stunted, leaves appear mosaic, blistering, deformation and small sizes. Fruit symptoms are uneven colouring. In the *FIGURE 4* is wild pumpkin infected with ZYMV. Symptoms are yellow mottling and green banding. (13)

FIGURE 4 Wild pumpkin infected with ZYMV as observed in field 25 (Photo by Maria Sydänmetsä)

1.2.3 *Watermelon mosaic virus* (WMV)

As CMV and ZYMV also WMV causes huge financial losses on yields. Virus is transmitted by aphids in a non-persistent manner. First details have been documented in 1956 but it hasn't been yet described complete even though it has been well known virus around the world.

Symptoms are mottling, blistering, chlorosis on leaves and plant stunting. Cross-protection, crop rotation and genetic resistance have been used against the virus. (21) The effect of the ZYMV is seen on the *Figure 5*.

Figure 5 Watermelon infected with WMV (22)

1.3 Enzyme-linked immunosorbent Assay (ELISA)

Samples were tested using ELISA method to find out whether the plant has been infected with CMV, WMV or ZYMV. ELISA is a laboratory technique which is used to detect and quantify proteins, antibodies, hormones or peptides in sample. Procedure consist of five steps, which are:

> *"1) Coat the microtiter plate wells with antigen; 2) block all unbound sites to prevent false positive results; 3) add primary antibody (e.g. rabbit monoclonal antibody) to the wells; 4) add secondary antibody conjugated to an enzyme (e.g. anti-mouse IgG); 5) reaction of a substrate with the enzyme to produce a coloured product, thus indication a positive reaction." (22)*

The most known ELISA applications are the HIV-test, home pregnancy test and allergy tests. Different methods can be used depending on what have been in-

vestigating. The typical ELISA formats are direct ELISA, indirect ELISA, sandwich ELISA and competition ELISA. ELISA has a high sensitivity and strong specificity. (23)

1.3.1 Double Antibody Sandwich ELISA (DAS-ELISA)

The less common type of ELISA is Sandwich ELISA, aka DAS-ELISA. Sandwich ELISA determine antigens between two layers of antibody, which are capture antibody and detection antibody. A monoclonal antibody is usually the detection antibody and a polyclonal is used as a capture antibody.

The steps are as follows:

1. *Prepare a surface to which a known quantity of capture antibody is bound.*
2. *Block any nonspecific binding sites on the surface.*
3. *Apply the antigen-containing sample to the plate.*
4. *Wash the plate, so that unbound antigen is removed.*
5. *A specific antibody is added, and binds to antigen (hence the 'sandwich': the Ag is stuck between two antibodies);*
6. *Apply enzyme-linked secondary antibodies as detection antibodies that also bind specifically to the antibody's Fc region (non-specific).*
7. *Wash the plate, so that the unbound antibody-enzyme conjugates are removed.*
8. *Apply a chemical that is converted by the enzyme into a color or fluorescent or electrochemical signal.*
9. *Measure the absorbency or fluorescence or electrochemical signal (e.g., current) of the plate wells to determine the presence and quantity of antigen. (24)*

Positive things on DAS-ELISA are that samples are easy to handle before analyse, because they do not have to be purified first. Also analyse is very sensitive comparing to indirect-ELISA.

1.3.2 Antibodies

Antibodies are proteins, which are part of the immune system. Their functions are to identify and neutralize viruses and bacteria. Each antibody has its own target. The target is called antigen. Antibody-protein is shape of the Y, so the

tips of the Y and the antigen have the same structure. Antibody and antigen are working like the lock and the key. (25)

The antibodies used on this experience were AS-0234 IgG; AS-0234 IgG-AP (against ZYMV), AS-0929 IgG; AS-0929 IgG-AP (against CMV) and AS-0203 IgG; AS-0203 IgG; AS-0203 IgG-AP (against WMV).

2 ON THE FIELD

Samples were collected from two regions called Coast and Dar es Salaam. On the Coast region the districts were Bagamoyo and Kibaha. The districts surveyed in Dar es Salaam were Kinondoni, Temeke and Ilala. The farms were chosen randomly by driving around and stopping when farms were visible or known. Also the help from local people was needed.

2.1 The farms

Farms were located as they are shown in the *FIGURE 6*. Farms were small farms and every farmer was asked permission to enter the farm and collect some leaves. The procedure was to find farm by looking, searching by car and asking local people. When the farm had been found, the permission to enter to the farm and collect samples was asked from the owner. Also farmers were asked a short questionnaire about farming and viruses.

FIGURE 6 The farms on the map

Some samples of wild host plants were also collected from nearby farms. Coordinates and pictures were taken on every farm using GPS. Samples were collected on two separate days. 11 farms were visited on February 17 and 10 farms and on March 1, 2016. There were farms without symptoms (*FIGURE 7*) and symptomatic ones (*FIGURE 8*).

First samples were collected around the Coast area from four pumpkin farms, five watermelon farms and two cucumber farms. Zucchini was not found on this area. Crop age was from one to two months.

Second day the samples were collected from Temeke and Ilala from four pumpkin farms, two watermelon farms and three cucumber farms. Zucchini was not found on this area either. Crop age was from two weeks to two months. Some samples were also collected from wild plants around the area.

FIGURE 7 A field with no symptomatic plants

FIGURE 8 A field with symptomatic plants

2.2 The samples

All together 21 farms were visited and from ten to sixteen samples were collected from each farm. All in all 115 samples were collected on the first day. Two of the samples were left out from the ELISA-test because of the broken baggage. 107 samples were collected on the second day. The farm number 20 were left out from the final ELISA-test. All together 222 collected samples were tested.

Farms were named from #1 to #10 and from #20 to #30, and each sample has its own number. For example sample number #6-2 means that sample was collected from the farm number 6 and it was the second collected sample. Each sample was documented, either with a mention no symptoms or describing symptoms.

Samples were collected randomly using “x”-pattern, five samples from corner to corner and five from another corner to another. 22 samples were collected on nearby farms from wild plants, which is 10% of all samples. Samples were

taken from the newest part of the plant, usually the latest grown leaves and were put on plastic sample extraction bags with the number of the sample written on bag (*FIGURE 9*). Samples were stored in cold box during the day and the evening they were put into the fridge +4 for the night.

FIGURE 9 Sample bag with filters (DSMZ Institute in Germany)

2.3 A short questionnaire of farmers knowledge on diseases

At the same time when the farms were visited, farmers were asked some questions about farming and diseases to gather some information about the background of the farmers and the fields. Questionnaire is shown as an *Appendix 1*, original in Swahili and translated version in English.

The purpose of the questionnaire was to find out if the farmers have noticed any diseases on the farm and if so, what they have done about it. And most importantly, if they have ever been thinking about giving up farming because of the diseases.

3 ELISA

The laboratory work was done using pest control laboratory in Mikocheni Agricultural Research Institute, Dar es Salaam, Tanzania. Buffers were prepared in transformation laboratory. This chapter is dived in four subchapters, the first one focuses on reagents, the second one on equipment, the third one on buffers and the fourth one on the ELISA work itself.

3.1 Reagents

The used reagents are shown in *TABLE 1* and ELISA kit components in *TABLE 2*.

TABLE 1 Reagents

Reagent	**Molecular formula**	**Manufacturer**	**LOT number**	**CAS number**
Sodium bicarbonate	$NaHCO_3$	Amresco Pure	0094C240	26628-22-8
Sodium Carbonate anhydrous	Na_2CO_3	Amresco Pure	3635C191	497-19-8
Natrium Chloride	NaCl	Saboor Traiding	7647-14-5	213761
Monobasic potassium phosphate	KH_2PO_4	Amresco Pure	2154C096	7778-77-0
Dibasic sodium phosphate	$Na_2HPO_4 \cdot 2H_2O$	BDH Analar		
Potassium chloride	KCl	BDH GPR	K26325337924	295944

Sodium Azide	NaN_3	Amresco Pure	0094C240	26628-22-8
PBS Tween$_{20}$		Amresco Pure	0134C141	9005-64-5
Diethanolamine	$C_4H_{11}NO_2$	Amresco Pure	0644C321	111-42-2
Polyvinylpyrrolidone	$(C_6H_9NO)_n$	Amresco Pure (HPG)	0754C369	9003-39-8
Bovine Serum Albumin		Sigma	SLBH4587V	9048-46-8
4-nitrophenyl phosphate disodium salt hexahydrate		Sigma	001387334	333338-18-4
Alkaline phosphatase yellow liquid substrate system		Sigma	SLBKI704V	P7998
Distilled Water	H_2O			

TABLE 2 DAS-ELISA kit

DAS-ELISA kit with AS-0234 (IgG, IgG-AP) against *Zucchini yellow mosaic virus*
PV-0466 (*Zucchini yellow mosaic virus*, positive control)
DAS-ELISA kit with AS-0929 (IgG, IgG-AP) against *Cucumber mosaic virus*
PV-0929 (*Cucumber mosaic virus*, positive control)
DAS-ELISA kit with AS-0203 (IgG, IgG-AP) against *Watermelon mosaic virus*
PV-0394 (*Watermelon mosaic virus*, positive control)

3.2 Equipment

Equipment of the disease control laboratory and molecular laboratory were used during the work. List of the equipment is shown in *TABLE 3*.

TABLE 3 Equipment

Weight	KERN Eg 220-3NM (lab?)
pH meter	Mettler Toledo Seven G028940
Pipettes	Fisherbrand Finnpipette C57771 (5-50 µl) Capp Bravo JH11016 (2-20 µl) Capp Bravo JH10139 (20-200 µl)
Incubator	Stuart S1500 Lasek R00102131

ELISA Plate Analyser	Micro plate read RT0030115GDM

3.3 Preparing buffers

Buffers were prepared using ELISA protocol by Leibniz-Institut (*Appendix 2*). All the containers have been rinsed with distilled water before using.

Coating buffer (pH 9.8)

- ✓ 1,59 g sodium carbonate (Na_2CO_3)
- ✓ 2,93 g sodium bicarbonate ($NaHCO_3$)
- ✓ 0,20 g sodium azide (NaN_3)

After weighting reagents they were dissolved in 900 ml distilled water. After that pH has been adjusted being 9.8 with 1M sodium hydroxide and made up to 1 litre.

PBS (pH 7.4) phosphate buffered saline

- ✓ 8.0 g sodium chloride (NaCl)
- ✓ 0.2 g monobasic potassium phosphate (KH_2PO_4)
- ✓ 1.15 g dibasic sodium phosphate (Na_2HPO_4)
- ✓ 0.2 g potassium chloride (KCl)
- ✓ 0.2 g sodium azide (NaN_3)

After weighting reagents have been dissolved in 900 ml distilled water. After that pH has been adjust being 7.4 and solution made up to 1 litre. This buffer was prepared twice, so the final amount of this buffer was 2 litre in two different containers.

PBS-Tween (PBST)

- ✓ PBS + 0.5 ml $Tween_{20}$ per litre

$Tween_{20}$ was pipetted 0.5 ml on both container with 1 litre of PBS.

Sample extraction buffer (pH 7.4)

- ✓ PBST + 2% PVP (polyvinyl pyrrolidone)

1000 ml x 0.02 = 20 g (2%)

20 g of PVP was added in one litre of PBST. Solution was separate in two different container, each of 500 ml.

Conjugate buffer

- ✓ PBST + 2% PVP + 0.2 egg albumin

Instead of egg albumin same amount of Bovine Serum Albumin was used. 1 g. of Bovine Serum Albumin was added in 500 ml of PBST + PVP.

Substrate buffer

- ✓ 97 ml diethanolamine
- ✓ 600 ml H_2O
- ✓ 0.2 g sodium azide (NaN_3)

Diethanolamine and sodium azide were measured in container, water was added up to 800 ml and pH were adjusted to 9.8 with hydrochloride. Water was added and made up to 1 litre.

All buffers were stored at 4°C.

3.4 Performing ELISA

The samples were collected on two different days. It is important to use fresh leaves when performing ELISA. Therefore ELISA test was done the next day after the samples were collected. The second part was executed the same way as the first part with couple of exceptions, which are found in chapters below.

3.4.1 The first ELISA test

All twelve ELISA plates were numbered from 1-12, first 1-4 were for ZYMV, next ones 5-8 were for WMV and 9-12 for CMV. Antibodies (AS-0234, AS-0929, AS-0203) were diluted in coating buffer using recommended dilutions 1:1000. Each coating buffer was prepared 80 ml with diluting 80 µl antibodies. Antibodies were added on ELISA-plates each well 200 µl. Plates were covered by foil and incubated in 37°C four hours. Plates were washed with PBST three times.

The first sample was weighed (0.2 g) and next ones were measured visually to have about the same amount of the leave as the measured one. The rest of the samples were stored in -80°C in paper bags inside a plastic bag. 3 ml extraction buffer was added in sample extraction bags and samples were grinded. Samples were added on plates pipetting 200 µl in each well. The buffer was added in the two wells and the positive control was added in the other two wells. The edge wells were not used. The sample map is shown on *Appendix 3*.

Plates were covered by foil and incubated over night at +4°C.

Plates were washed three times with PBST after overnight incubation. Enzyme conjugate was prepared in conjugate buffer (recommended dilution is given in the protocol). Each conjugate buffer was measured 40 ml and added 40 µl enzyme, except AS-0203 IgG-AP was added 80 µl. Each well was added 200 µl enzyme conjugate. Plates were covered by foil and incubated at +37°C three hours. Plates were washed three times with PBST.

The final step was to add 200 µl of substrate in each well. Unlike it was written in protocol, the used substrate was Alkaline Phosphatase Yellow liquid substrate system for ELISA. Plates were covered and incubated 37°C.

After 30 minutes the incubation plates were read by ELISA reader by wavelength 405 nm. Plates were put back in the incubator and were read again after 120 minutes. Visual observation was made as well and documented.

3.4.2 The second ELISA test

The second test was made with the same equipment and reagents as in the first one. The plates were numbered from 1-12, first four plates being CMV, next four ZYMV and last four WMV. The sample map is shown in *appendix 3*.

On the first round results of the WMV and CMV plates were yellowish probably because of the too high enzyme concentrations, this time the following concentrations were used:

Recommended dilutions AS-0929 IgG-AP were 1:1000, but used 1:1250. Recommended dilutions AS-0203 IgG-AP were 1:500, but used 1:600.

This time the substrate was prepared as the protocol suggested. 4-nitrophenyl phosphate disodium salt hexahydrate was added just before the substrate was used.

Otherwise everything was performed as in the chapter 3.4.1.

4 RESULTS

All together 222 samples were tested with antibodies raised against ZYMV, CMV and WMV. ELISA plates were read by visually and using Elisa plate reader. Subchapter 4.1 shows results of the visual observation and subchapter 4.2 shows ELISA reader results. The final results, which are based on both visual and reader results, are shown on subchapter 4.3. The last subchapter 4.4 summarizes the questionnaire results.

4.1 ELISA Visual results

Visual results are based on visual observation of the plates. Plates were visually observed after 30 minutes and again after 120 minutes. The results were documented and plates were photographed. Pictures of the plates are shown in *appendix 4*. Samples were collected together 224 but two of them were left out from the testing. 222 samples were tested with antibodies raised against ZYMV, CMV and WMV.

ZYMV plates were easy to read visually. All positive controls were shown as yellow and buffers were clear. Positive ones were brightly yellow. 21 samples out of 222 were positive with ZYMV.

The results on the CMV with the first 113 samples were little bit yellowish but the positive controls were easily observed. Buffers were clear also and positive samples stood out. The next 109 samples were little less yellowish, probably because of the changes with concentration of enzyme buffer, and positive ones were more easily readable. 22 samples out of 222 were positive with CMV.

The results on the first 113 WMV samples were all little bit yellowish, but positive controls and positive samples were still observable. Also buffers were clear. The results of the 109 samples collected from 10 fields in Dar es Salaam are not shown because the colour development was rapid and positive samples

could not be differentiated from negative ones. Result from the first 113 samples were counted, therefore 19 samples out of 113 were tested positive with WMV.

4.2 ELISA Reader results

The plates were read by ELISA reader after 30 minutes and again after 120 minutes. The colouring already began in 30 minutes, but the difference was more visible after 120 minutes. The results of 30 minutes and 120 minutes are shown in *Appendix 5*.

Not having a negative control the buffer has been used as an indicator of negative ones and positive controls were indicator as estimating positive ones. The plates without too much yellowish colour the positive samples were easily read. With other ones the results were compared with buffer and positive control.

Samples were marked as positive ones if the value of the absorbance was higher than positive control or much closer to the positive control than value of the buffer. Even though if the sample has not shown positive on visual observation it has been marked as a positive one if the reader results are clearly favouring positive results.

The results of the ELISA reader in ZYMV were shown 25 positive samples out of 222, which is four more than visual observation. The results of CMV were 24 positive ones out of 222. Three samples were not showing positive signs on visually but according to the ELISA reader they are positive. One sample were visually yellowish, but reader shows no significant difference with negative ones.

The results of the ELISA reader in WMV were shown 21 positive samples. Together three of the samples were shown positive, even they were not clear on visual observation. On the other hand, one sample was shown as positive by visual looking but according to the ELISA reader it is not positive one. 109 were not readable meaning there were 21 positive ones out of 113.

Results indicates that ELISA Reader is more sensible showing positive results than it is observed visually. Observing results only by visually can mean that some positive ones stay unnoticed. Together there were 70 positive ones, 8 of them were found out only by ELISA Reader.

4.3 Results

In all 234 samples were collected from 21 different farms including some wild plants which were collected nearby farms. Two samples and one farm were left out, therefore in the end 222 samples from 20 farms were tested with antibodies raised against ZYMV, CMV and WMV.

27% of the samples were infected by CMV, ZYMV or WMV. There were only two farms (10%), where were no symptoms and no infected plants found. Farms were infected range from 0% to 80%. The one sample were tested positive with all three viruses, the sample was asymptomatic. 12% infected samples were positive with two viruses.

Cucumber samples were collected 53, pumpkin samples 81, watermelon samples 65 and 23 from wild plant. 43% wild plants, 30% pumpkin samples, 26% cucumber samples and 17% watermelon plants were tested positive. All of the 59 infected plants 39% were tested positive with CMV, and 41% with ZYMV. WMV were tested positive in 21 samples (36%). All viruses appear in all types of collected plants.

Wild plants are alternative hosts for viruses and can cause viruses transmit to the vegetables. 21 samples of wild plants were collected nearby farms including wild cucumber (*Cucumis hystrix*), wild pumpkin (*Curcubita foetidissima*), dodoki (*Luffa eagyptiaca*), cowpea (*Vigna unguiculata*) and matunda pori. 40% were tested positive to WMV, ZYMV or CMV, which is surprisingly big percentage.

Results are shown on the two tables, *TABLE 4* represents the percentage of the infected plants in each farm and *TABLE 5* positive results on each samples. The first column express the farm, the second one the plant, the third one the

sample number and the fourth one the symptoms. Description of the symptoms are shown on below table. The last column shows if the sample is positive with CMV, ZYMV or WMV.

TABLE 4 Percentage results of the infected plants

Farm	Samples	Positive	Positive %
1	10	5	50 %
2	10	8	80 %
3	10	1	10 %
4	10	5	50 %
5	10	0	0 %
6	10	4	40 %
7	10	1	10 %
8	10	3	30 %
9	12	4	33 %
10	10	1	10 %
11	12	3	25 %
21	11	2	18 %
22	10	0	0 %
23	10	0	0 %
24	10	1	10 %
25	11	2	18 %
26	12	5	42 %
27	16	2	13 %
28	11	6	55 %
29	10	5	50 %
30	10	1	10 %

TABLE 5 Samples with results

				Positive results		
Field	Crop	Sample	Symptoms*	ZYMV	WMV	CMV
#1	pumpkin	1	M	X		
#1	pumpkin	2	M			X
#1	pumpkin	3				
#1	pumpkin	4				X
#1	pumpkin	5				
#1	pumpkin	6				
#1	pumpkin	7				
#1	pumpkin	8			X	X
#1	pumpkin	9				

#1	pumpkin	10			X	X
#2	cucumber	1	Gb		X	X
#2	cucumber	2			X	X
#2	cucumber	3				
#2	cucumber	4		X	X	X
#2	cucumber	5			X	
#2	cucumber	6				X
#2	cucumber	7				X
#2	cucumber	8				
#2	cucumber	9				X
#2	cucumber	10	M			X
#3	pumpkin	1	Lg	X		
#3	pumpkin	2				
#3	pumpkin	3				
#3	pumpkin	4				
#3	pumpkin	5				
#3	pumpkin	6				
#3	pumpkin	7				
#3	pumpkin	8				
#3	pumpkin	9				
#3	pumpkin	10				
#4	pumpkin	1	M			
#4	pumpkin	2	Lg			
#4	pumpkin	3				X
#4	pumpkin	4				
#4	pumpkin	5	Ym			
#4	pumpkin	6			X	
#4	pumpkin	7	M			
#4	pumpkin	8		X		
#4	pumpkin	9			X	
#4	pumpkin	10			X	
#5	watermelon	1				
#5	watermelon	2				
#5	watermelon	3				
#5	watermelon	4				
#5	watermelon	5				
#5	watermelon	6				
#5	watermelon	7				
#5	watermelon	9				
#5	watermelon	10				
#6	pumpkin	1			X	
#6	pumpkin	2				

#6	pumpkin	3					
#6	pumpkin	4					
#6	pumpkin	5			X		
#6	pumpkin	6			X		
#6	pumpkin	7	M				
#6	pumpkin	8	m, ym				
#6	pumpkin	9	M				
#6	pumpkin	10	R	X			
#7	watermelon	1					
#7	watermelon	2			X		
#7	watermelon	3					
#7	watermelon	4					
#7	pumpkin	5					
#7	watermelon	6					
#7	watermelon	7					
#7	watermelon	8					
#7	watermelon	9					
#7	watermelon	10					
#8	watermelon	1	Y				
#8	watermelon	2	M		X		
#8	watermelon	3			X		
#8	watermelon	4	y, m				
#8	watermelon	5					
#8	watermelon	6	Df				
#8	watermelon	7	lg, df				X
#8	watermelon	8	ym, m				
#8	watermelon	9	ym, m				
#8	watermelon	10	Df				
#9	watermelon	1		X	X		
#9	watermelon	2	Df				
#9	watermelon	3					
#9	watermelon	4	Yp	X			
#9	watermelon	5	Y				
#9	watermelon	6	Y				
#9	watermelon	7	y, m				
#9	watermelon	9					
#9	watermelon	10	Y				
#9	wild cucurbit	11	m, yp	X			
#9	wild cucurbit	12	m, yp		X		
#10	watermelon	1					
#10	watermelon	2	Df				
#10	watermelon	3					

#10	watermelon	4				
#10	watermelon	5				
#10	watermelon	6	Df			
#10	watermelon	7				
#10	watermelon	8				
#10	watermelon	9				
#10	watermelon	10				
#10	wild cucumber	11			X	
#11	cucumber	1				
#11	cucumber	2			X	
#11	cucumber	3				
#11	cucumber	4				
#11	cucumber	5			X	
#11	cucumber	6			X	
#11	pumpkin	7	Df			
#11	cucumber	8				
#11	cucumber	9				
#11	cucumber	10				
#11	cucumber	11				
#11	cucumber	12				
#21	pumpkin	1		X	NA	
#21	pumpkin	2			NA	
#21	pumpkin	3			NA	
#21	pumpkin	4			NA	
#21	pumpkin	5			NA	
#21	pumpkin	6	Y	X	NA	
#21	pumpkin	7	M		NA	
#21	pumpkin	8	Lg		NA	
#21	pumpkin	9			NA	
#21	pumpkin	10	M		NA	
#21	wild pumpkin	11			NA	
#22	watermelon	1	M		NA	
#22	watermelon	2	M		NA	
#22	watermelon	3			NA	
#22	watermelon	4			NA	
#22	watermelon	5			NA	
#22	watermelon	6			NA	
#22	watermelon	7			NA	
#22	watermelon	8			NA	
#22	watermelon	9	M		NA	
#22	watermelon	10	Lg		NA	
#23	cucumber	1			NA	

#23	cucumber	2			NA	
#23	cucumber	3			NA	
#23	cucumber	4			NA	
#23	cucumber	5			NA	
#23	cucumber	6			NA	
#23	cucumber	7			NA	
#23	cucumber	8			NA	
#23	cucumber	9			NA	
#23	cucumber	10			NA	
#24	pumpkin	1			NA	
#24	pumpkin	2			NA	
#24	pumpkin	3			NA	
#24	pumpkin	4			NA	
#24	pumpkin	5			NA	
#24	pumpkin	6			NA	
#24	pumpkin	7			NA	
#24	pumpkin	8			NA	
#24	pumpkin	9			NA	X
#24	wild cucumber	10			NA	
#25	pumpkin	1			NA	
#25	pumpkin	2			NA	
#25	pumpkin	3			NA	
#25	pumpkin	4			NA	
#25	pumpkin	5	lg, m	X	NA	
#25	pumpkin	6			NA	
#25	pumpkin	7			NA	
#25	pumpkin	8			NA	
#25	pumpkin	9			NA	
#25	pumpkin	10			NA	
#25	wild pumpkin	11	y, gb, m	X	NA	
#26	dodoki	1			NA	
#26	dodoki	2			NA	
#26	dodoki	3			NA	
#26	dodoki	4			NA	X
#26	wild cucumber	5	Ys	X	NA	
#26	wild cucumber	6	Ys		NA	
#26	wild cucumber	7		X	NA	X
#26	wild cucumber	8		X	NA	
#26	wild cucumber	9		X	NA	
#26	wild cucumber	10			NA	
#26	matunda poli	11			NA	
#26	matunda poli	12			NA	

#27	cucumber	1	M	X	NA	
#27	cucumber	2	M	X	NA	
#27	cucumber	3			NA	
#27	cucumber	4			NA	
#27	cucumber	5	ys, gb		NA	
#27	cucumber	6	Ys		NA	
#27	cucumber	7	Ys		NA	
#27	cucumber	8			NA	
#27	cucumber	9	Ys		NA	
#27	cucumber	10	Ys		NA	
#27	cucumber	11			NA	
#27	cucumber	12			NA	
#27	wild cucumber	13			NA	
#27	wild cucumber	14			NA	
#27	wild cucumber	15			NA	
#27	wild cucumber	16			NA	
#28	pumpkin	1			NA	
#28	pumpkin	2	cl, se		NA	
#28	pumpkin	3			NA	
#28	pumpkin	4	Yp		NA	X
#28	pumpkin	5	cl, se		NA	
#28	pumpkin	6			NA	
#28	pumpkin	7	cl, se, yp		NA	X
#28	pumpkin	8	M		NA	X
#28	pumpkin	9			NA	X
#28	pumpkin	10			NA	X
#28	wild cowpie	11			NA	X
#29	watermelon	1	cl, gb		NA	
#29	watermelon	2			NA	
#29	watermelon	3	M		NA	
#29	watermelon	4	Gs	X	NA	
#29	watermelon	5	Cl	X	NA	
#29	watermelon	6	cl, m	X	NA	
#29	watermelon	7	cl		NA	
#29	watermelon	8		X	NA	
#29	watermelon	9	M	X	NA	X
#29	watermelon	10	cl, m, df		NA	
#30	cucumber	1	M	X	NA	
#30	cucumber	2	gb, m		NA	
#30	cucumber	3	m, gs		NA	
#30	cucumber	4	m, cl		NA	
#30	cucumber	5			NA	
#30	cucumber	6	m, gb		NA	

#30	cucumber	7	cl, m	NA
#30	cucumber	8	m, y	NA
#30	cucumber	9	cl	NA
#30	cucumber	10	m, cl, gb	NA

*cl=curly leaves df=deformed gb=green banding gs=green spots lg=light greenish m=mosaic r=rugosity se=sharp edges y=yellowish ym=yellow mottling yp=yellow pattern ys= yellow spots

4.4 Farmers experiences of viruses

In order to gather information on the farmers the questionnaire was filled on 20 farms by farmers. The purpose of the questionnaire was to find out if the farmers have noticed any diseases on the farm and if so, what they have done.

70% of the farmers were male and the most common age was 31-45 years. 70% of the farmers had primary school education, only 30% had been in secondary school. 75% had experience of farming under five years.

Most of the farmers had seen diseases on the farm, as much as 85%. Answering the question what they did when they saw diseases on the farm, 85% answered they used pesticides. 20% told that they had thought about giving up farming due the diseases and economical losses. One of them even gave up for a while. However, most of them who has not thought about giving up pointed out, that the farming is their only livelihood so they just keep going.

The farmers who had chosen to grow watermelons said that even though the seeds are expensive, the profit form watermelons is good. Pumpkins and cucumber are less profitable but cheaper to grow. 90% of the farmers bought seeds from the Agro shop, one farmer got them from home and one farmer used previous seeds. The farm which used previous seeds was severe symptomatic but only one sample was tested positive in ZYMV.

Overall farmers were willing to answer the questions and showing the fields. They were keen to learn about the viruses and diseases and how the symptoms show on plants.

5 DISCUSSION AND CONCLUSION

All three viruses (CMV, ZYMV and WMV) affect crops around the world causing huge economical losses. Establishing the incidence and distribution of viruses on the farms of watermelon, cucumber and pumpkin results show that all three viruses are found in all three varieties of family *cucurbitaceae*. 26% of the collected samples were tested positive, which proves that the viruses have spread out widely on the Coast and Dar es Salaam Regions in Tanzania.

Symptoms were visually seen on the farms. The symptoms did vary between plants, but not between species. Most common symptoms were mosaic, yellow mottling and rugosity on leaves. Also there were curly leaves, yellow spots and green bandings. Some plants were deformed. The reason of some symptoms, for example yellow leaves can be caused by different diseases or lack of water, but mosaic and yellow mottling are usually caused by viruses.

All the viruses caused symptoms in all three plants, but it seems like symptoms were more conspicuous on watermelon farms. Asymptomatic watermelon farms did not have viruses and symptomatic ones did have 30-50%. On the watermelon farms with symptomatic plants, the symptoms were quite severe. On other hand one cucumber farm seemed to have no symptoms on the leaves, but all three viruses were found and 70% of the samples was tested positive. And on this particular farm one cucumber sample without any symptoms were tested positive for all three viruses.

Some of the infected pumpkins showed the symptoms but some of them not. One cucumber farm was widely symptomatic with severe damage, but only one sample tested positive for ZYMV. This indicates there are yet some other viruses causing similar symptoms.

There were two farms without symptomatic plants and no viruses found. Both were clearly separated from surroundings with soil area. That can be the reason, that there has not been contamination with diseased wild plants around.

One farm had two fields close to each other, first one was symptomatic and tested positive, other one was asymptomatic and samples showed negative. Between these two fields there were couple of meters of soil. By isolating the farm of the surrounding wild plants (alternative hosts) or perhaps cutting and destroying them around farms could prevent viruses from transmitting to the vegetables. This could be something which might be worth to research more.

Some co-infections were also found in this study. Co-infections of CMV (*Cucumovirus)* with both ZYMV (*Potyvirus*) and WMV (*Potyvirus*) were observed. The most usual combination was CMV and WMV. In one case all three viruses were found on a plant. Therefore there is a possibility for synergism, even though it has not been establish yet.

It is not a surprise that CMV, ZYMV and WMV have been found around Dar es Salaam and Coast regions in Tanzania considering all three viruses have spread widely all over the world. The fields were symptomatic and viruses cause economical losses to the farmers, because of the weakened quality of the vegetables. The crops are main source of income to the farming families and crop failure means reduced income.

The farmers should have knowledge to recognize symptoms of different viruses. As the questionnaire showed they realize if there is something wrong with the crop, but they do not know what the problem is and what to do with it. The education of the farmers is highly recommendable.

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DODOSO FUPI

Ndungu mkulima dodoso hili fupi linalenga kukusanya habari kwa ufupi juu ya kilimo cha mazao jamii ya maboga (cucurbits). Habari itakayotolewa itakuwa ni siri na itatumika tu kwa faida za kitaaluma na siyo kibiashara.

Mkulima

1. Jina la Mkulima (optional)
2. Umri
3. Elimu
4. Jinsia
5. Uzoefu wa kilimo cha maboga (miaka)

Mbegu na magonjwa

1. Mbegu inayotumika upatikana wapi
2. Bei ya mbegu kwa eka moja ni shillingi ngapi
3. Je umeona magonjwa kwenye shamba lako
4. Ukiona magonjwa unafanya nini
    a. Natumia dawa
    b. Napumizisha shamba
    c. Sifanyi kitu

5. Umewahi kukatishwa tamaa na magonjwa na kuacha kulima?

6. Kwa nini unalima aina ya maboga unayolima?

Ashante

Short questionnaire

This short questionnaire for the farmers focuses briefly on gathering information about the agricultural products of the cucurbits family. Information will be confidential and used only purpose of the professional work and will not be used in commercial matters.

Farmer

6. Name of the farmer (optional)
7. Age
8. Education
9. Gender
10. Experience of the farming (years)

Seeds and diseases

7. Where are the seeds from
8. Price of the seeds per one acre in shillings
9. Have you seen diseases on your field
10. When you see diseases what do you do
    a. I use pesticides
    b. I let field to fallow
    c. I don’t do anything

11. Have you ever given up farming due to the diseases?

12. Why do you grow this type of curcubits?

Thank you

Leibniz-Institut
DSMZ-Deutsche Sammlung von
Mikroorganismen und Zellkulturen GmbH

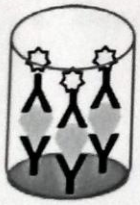
DSMZ

Double Antibody Sandwich ELISA (DAS-ELISA)

Our ELISA reagents are optimized using greiner bio-one microplates, medium binding.
Before opening the tubes containing coating antibody (IgG) and IgG-AP- Conjugate please spin down all the liquid by a short centrifugation (approx. 3000rpm for a few seconds).

1. Dilute specific antibody in coating buffer (recommended dilution see delivery note and tube); i.e. 20µl in 20 ml buffer at a recommended dilution of 1:1000 or 40µl in 20 ml buffer at a recommended dilution of 1:500. Add 200µl to each well of the microtiter plate.
2. Cover the plates and incubate at 37 °C for 2- 4 h.
3. Wash plate with PBS-Tween using wash bottle, soak for a few minutes and repeat washing two times. Blot plates by tapping upside down on tissue paper.

4. Extract samples 1:20 (w/v) in extraction buffer. Add 200 µl aliquots of the test sample to duplicate wells.
5. Cover the plates and incubate overnight at 4 °C.
6. Wash three times as in step 3.



7. Add 200 µl enzyme conjugate, recommended dilution is given in the delivery note, in conjugate buffer.
8. Cover the plates and incubate at 37 °C for 2- 4 hours.
9. Wash three times as in step 3.

10. Add 200 µl aliquots of freshly prepared substrate (1 mg /ml para- nitrophenyl- phosphate in substrate buffer) to each well.
11. Cover the plate and incubate at 37°C for 30-60 min, or as long as necessary to obtain clear reactions.
12. Assess results by:
    a) Visual observation
    b) Spectrophotometric measurement of absorbance at 405 nm

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Buffers used in ELISA

1. ***Coating buffer (pH 9.6)***

   1.59 g sodium carbonate (Na_2CO_3)
   2.93 g sodium bicarbonate ($NaHCO_3$)
   0.20 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 9.6 with HCl and make up to 1 l.

2. ***PBS (pH 7.4) phosphate buffered saline***

   8.0 g sodium chloride (NaCl)
   0.2 g monobasic potassium phosphate (KH_2PO_4)
   1.15 g dibasic sodium phosphate (Na_2HPO_4)
   0.2 g potassium chloride (KCl)
   0.2 g sodium azide (NaN_3)
   Dissolve in 900 ml H_2O, adjust pH to 7.4 with NaOH or HCl and make up to 1 l.

3. ***PBS-Tween (PBST)***

   PBS + 0.5 ml Tween 20 per liter

4. ***Sample extraction buffer (pH 7.4)***

   PBST + 2% PVP (e.g. Serva PVP-15 polyvinyl pyrrolidone)

5. ***Sample extraction buffer (pH 8.5) for Begomoviruses***

   0.05 M Tris containing 0.06 M sodium sulfite, pH 8.5

6. ***Conjugate buffer***

   PBST + 2% PVP + 0.2% egg albumin (e.g. Sigma A-5253)

7. ***Substrate buffer***

   97 ml diethanolamine
   600 ml H_2O
   0.2 g sodium azide (NaN_3)
   Adjust to pH 9.8 with HCl and make up to 1 liter with H_2O

Buffers can be stored at 4 ° C for at least 2 months. Warm to room temperature before use.

ELISA Troubleshooting

1. *No color development*

a) Did you omit any steps?
b) Did you use the correct buffer for each step?
c) Is your enzyme OK? Serum OK?
d) Is your positive control homologous to antiserum (IgG)?

Recommendations - Do a titration plate. Use a reliable positive control in each plate. Pretest enzyme conjugate on substrate.

2. *Nonspecific color development*

a) If in edge wells only:
- Make sure the humidity in the incubator is sufficiently high.
- If this does not help, don't use edge or border wells, fill with buffer only.

b) If in whole plate:
- incomplete washing
- old substrate
- use recommended ELISA plate (greiner medium binding)
- error in loading sequence

Recommendations - Use reliable negative control in each plate.
Use fresh substrate and check for spontaneous color change. Cover plates while incubating. Check pH of the buffers used.

c) Some wells with inconsistent or unexpected reactions
- incomplete washing
- error in loading test antigens
- spillage between wells

Recommendations - Use extra wash step, handle plates carefully with lids on, use predetermined loading pattern before loading. Blot top of plate after rinsing.

3. *Color development very rapid; some color in healthy samples*

a) Enzyme conjugate concentration too high
b) Substrate concentration too high

Recommendations - Use enzyme conjugate and substrate concentrations that will give $OD_{405\ nm}$ of about 1.0 in 30 to 60 min with good antigen source.

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Plate 1, 5 and 9

x	x	X	x	x	x	x	x	x	x	x	x
x	#1-2	#1-2	#1-3	#1-3	#1-4	#1-4	#1-6	#1-6	#1-7	#1-7	x
x	Positive	Positive	Buffer	Buffer	#1-8	#1-8	#1-9	#1-9	#1-10	#1-10	x
x	#6-8	#6-8	#6-7	#6-7	#10-5	#10-5	#6-6	#6-6	#6-5	#6-5	x
x	#10-4	#10-4	#10-3	#10-3	#10-2	#10-2	#10-1	#10-1	#6-4	#6-4	x
x	#6-3	#6-3	#6-2	#6-2	#12-9	#12-9	#11-1	#11-1	#10-11	#10-11	x
x	#5-10	#5-10	#9-11	#9-11	#2-6	#2-6	#4-3	#4-3	#9-3	#9-3	x
x	x	X	x	x	x	x	x	x	x	x	x

Plate 2, 6 and 10

x	x	X	x	x	x	x	x	x	x	x	x
x	#9-2	#9-2	#9-1	#9-1	#8-1	#8-1	#7-7	#7-7	#7-10	#7-10	x
x	Positive	Positive	Buffer	Buffer	#5-3	#5-3	#4-1	#4-1	#6-1	#6-1	x
x	#1-1	#1-1	#9-7	#9-7	#9-6	#9-6	#4-2	#4-2	#9-5	#9-5	x
x	#9-4	#9-4	#8-10	#8-10	#7-4	#7-4	#9-12	#9-12	#2-10	#2-10	x
x	#8-7	#8-7	#11-8	#11-8	#2-9	#2-9	#2-8	#2-8	#2-7	#2-7	x
x	#4-6	#4-6	#11-11	#11-11	#11-7	#11-7	#11-12	#11-12	#8-4	#8-4	x
x	x	X	x	x	x	x	x	x	x	x	x

Plate 3, 7 and 11

x	x	X	x	x	x	x	x	x	x	x	x
x	#8-5	#8-5	#11-10	#11-10	#8-3	#8-3	#8-6	#8-6	#8-2	#8-2	x
x	Positive	Positive	Buffer	Buffer	#11-2	#11-2	#11-5	#11-5	#11-3	#11-3	x
x	#12-6	#12-6	#4-10	#4-10	#4-4	#4-4	#7-9	#7-9	#3-8	#3-8	x
x	#2-1	#2-1	#7-5	#7-5	#2-2	#2-2	#8-8	#8-8	#7-2	#7-2	x
x	#7-6	#7-6	#4-5	#4-5	#7-3	#7-3	#7-1	#7-1	#2-5	#2-5	x
x	#5-2	#5-2	#5-5	#5-5	#7-8	#7-8	#3-9	#3-9	#2-3	#2-3	x
x	x	X	x	x	x	x	x	x	x	x	x

Plate 4, 8 and 12

x	x	X	x	x	x	x	x	x	x	x	x
x	#5-4	#5-4	#2-4	#2-4	#9-9	#9-9	#8-9	#8-9	#9-10	#9-10	x
x	Positive	Positive	Buffer	Buffer	#3-3	#3-3	#4-7	#4-7	#3-4	#3-4	x
x	#3-6	#3-6	#4-9	#4-9	#3-2	#3-2	#3-7	#3-7	#3-10	#3-10	x
x	#6-10	#6-10	#10-10	#10-10	#10-9	#10-9	#10-8	#10-8	#10-6	#10-6	x
x	#10-7	#10-7	#6-9	#6-9	#1-5	#1-5	#4-8	#4-8	#3-5	#3-5	x
x	#3-1	#3-1	#5-6	#5-6	#5-7	#5-7	#5-9	#5-9	#5-1	#5-1	x
x	x	X	x	x	x	x	x	x	x	x	x

Plates 1, 5 and 9

x	x	X	x	x	x	x	x	x	x	x	x
x	#26-8	#26-8	#27-2	#27-2	#27-4	#27-4	#24-7	#24-7	#24-1	#24-1	x
x	Positive	Positive	Buffer	Buffer	#30-6	#30-6	#24-6	#24-6	#24-8	#24-8	x
x	#30-7	#30-7	#28-10	#28-10	#24-4	#24-4	#27-3	#27-3	#24-3	#24-3	x
x	#24-2	#24-2	#22-10	#22-10	#22-4	#22-4	#22-3	#22-3	#23-10	#23-10	x
x	#21-9	#21-9	#22-2	#22-2	#29-7	#29-7	#29-6	#29-6	#29-1	#29-1	x
x	#28-11	#28-11	#27-16	#27-16	#28-8	#28-8	#25-10	#25-10	#27-10	#27-10	x
x	x	X	x	x	x	x	x	x	x	x	x

Plates 2, 6 and 10

1	2	3	4	5	6	7	8	9	10	11	12
x	x	X	x	x	x	x	x	x	x	x	x
x	#21-11	#21-11	#23-1	#23-1	#22-5	#22-5	#24-9	#24-9	#30-3	#30-3	x
x	Positive	Positive	Buffer	Buffer	#30-5	#30-5	#23-6	#23-6	#24-10	#24-10	x
x	#26-7	#26-7	#21-2	#21-2	#22-7	#22-7	#23-4	#23-4	#22-1	#22-1	x
x	#22-6	#22-6	#22-8	#22-8	#26-6	#26-6	#23-3	#23-3	#23-9	#23-9	x
x	#23-2	#23-2	#26-4	#26-4	#25-9	#25-9	#26-2	#26-2	#24-5	#24-5	x
x	#30-1	#30-1	#23-5	#23-5	#23-7	#23-7	#23-8	#23-8	#25-11	#25-11	x
x	x	x	x	x	x	x	x	x	x	x	x

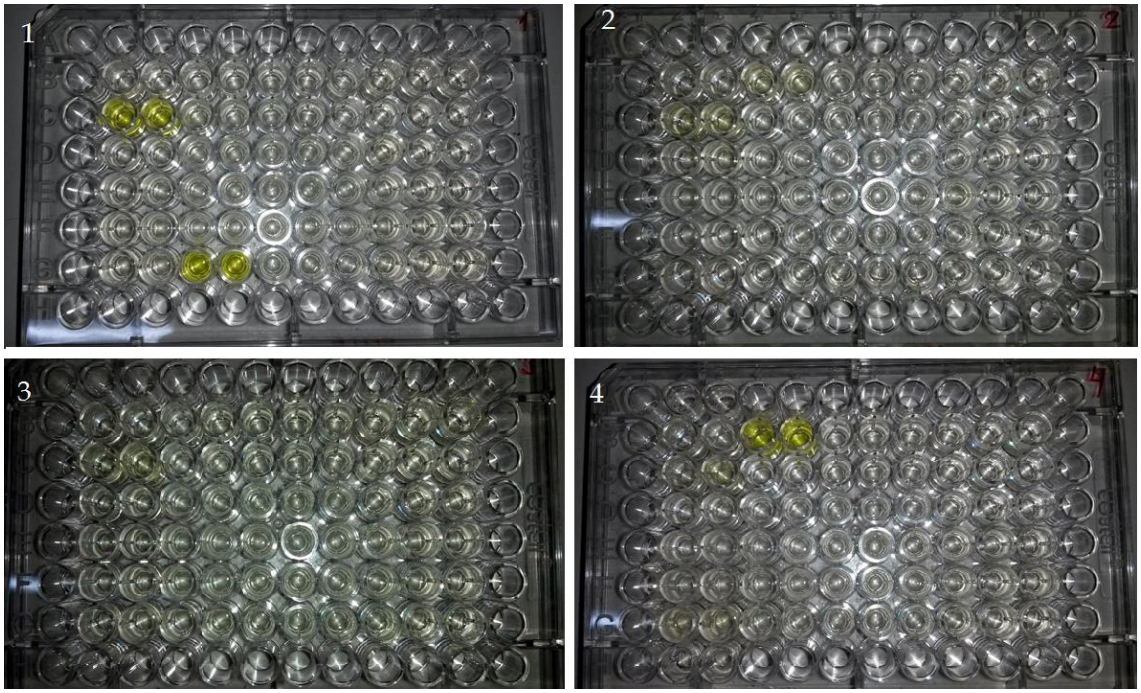
Plates 3, 7 and 11

1	2	3	4	5	6	7	8	9	10	11	12
x	x	x	x	x	x	x	x	x	x	x	x
x	#29-5	#29-5	#27-11	#27-11	#25-4	#25-4	#21-5	#21-5	#25-1	#25-1	x
x	Positive	Positive	Buffer	Buffer	#21-4	#21-4	#30-9	#30-9	#29-8	#29-8	x
x	#28-7	#28-7	#27-1	#27-1	#27-13	#27-13	#27-15	#27-15	#27-14	#27-14	x
x	#27-9	#27-9	#28-1	#28-1	#28-4	#28-4	#28-2	#28-2	#28-3	#28-3	x
x	#28-5	#28-5	#28-6	#28-6	#27-5	#27-5	#27-12	#27-12	#27-8	#27-8	x
x	#22-9	#22-9	#25-3	#25-3	#21-10	#21-10	#25-6	#25-6	Buffer	Buffer	x
x	x	x	x	x	x	x	x	x	x	x	x

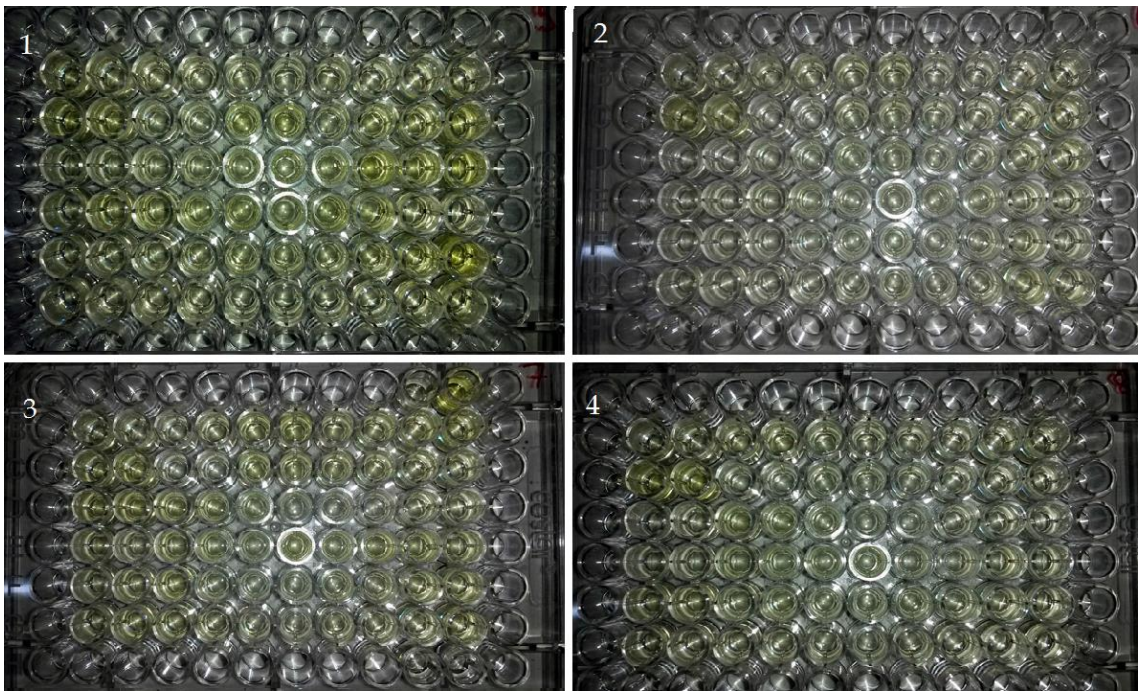
Plates 4, 8 and 12

1	2	3	4	5	6	7	8	9	10	11	12
x	x	x	x	x	x	x	x	x	x	x	x
x	#26-5	#26-5	#25-8	#25-8	#27-7	#27-7	#26-9	#26-9	#21-7	#21-7	x
x	Positive	Positive	Buffer	Buffer	#27-6	#27-6	#26-5	#26-5	#26-3	#26-3	x
x	#21-6	#21-6	#26-1	#26-1	#30-2	#30-2	#28-9	#28-9	#30-4	#30-4	x
x	#29-3	#29-3	#29-10	#29-10	#29-2	#29-2	#29-4	#29-4	#21-8	#21-8	x
x	#25-7	#25-7	#30-10	#30-10	#21-1	#21-1	#25-2	#25-2	#25-5	#25-5	x
x	#30-8	#30-8	#21-3	#21-3	#29-9	#29-9	#26-11	#26-11	#26-12	#26-12	x
x	x	x	x	x	x	x	x	x	x	x	x

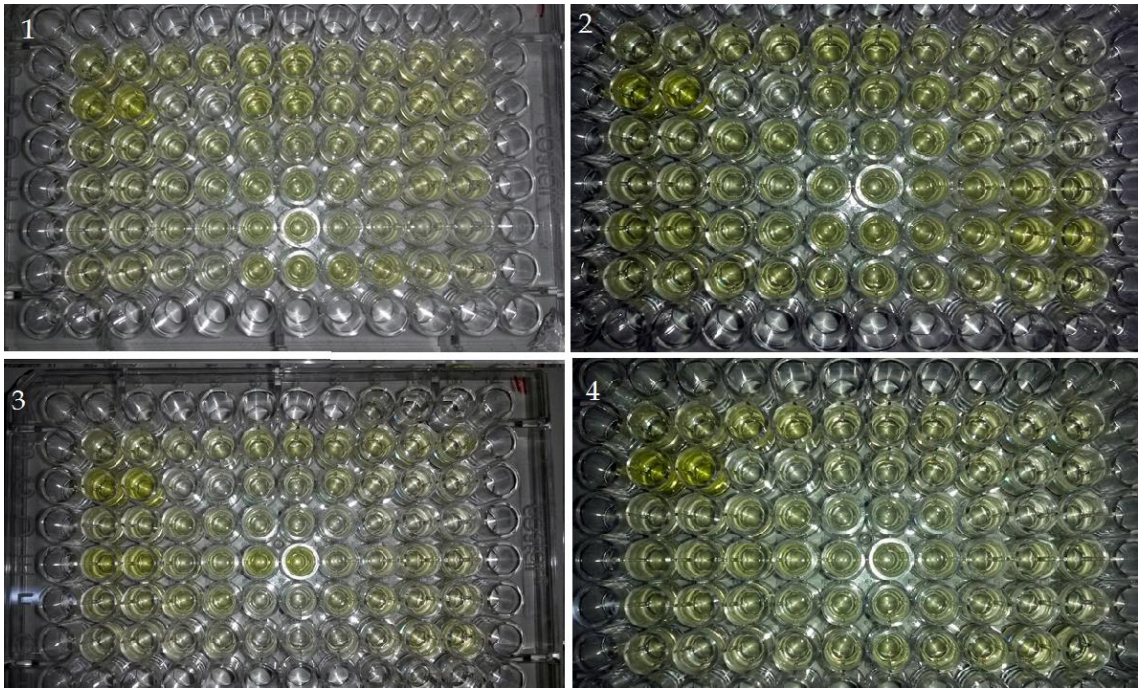
ZYMV-plates (1-4) from farms #1 - #11



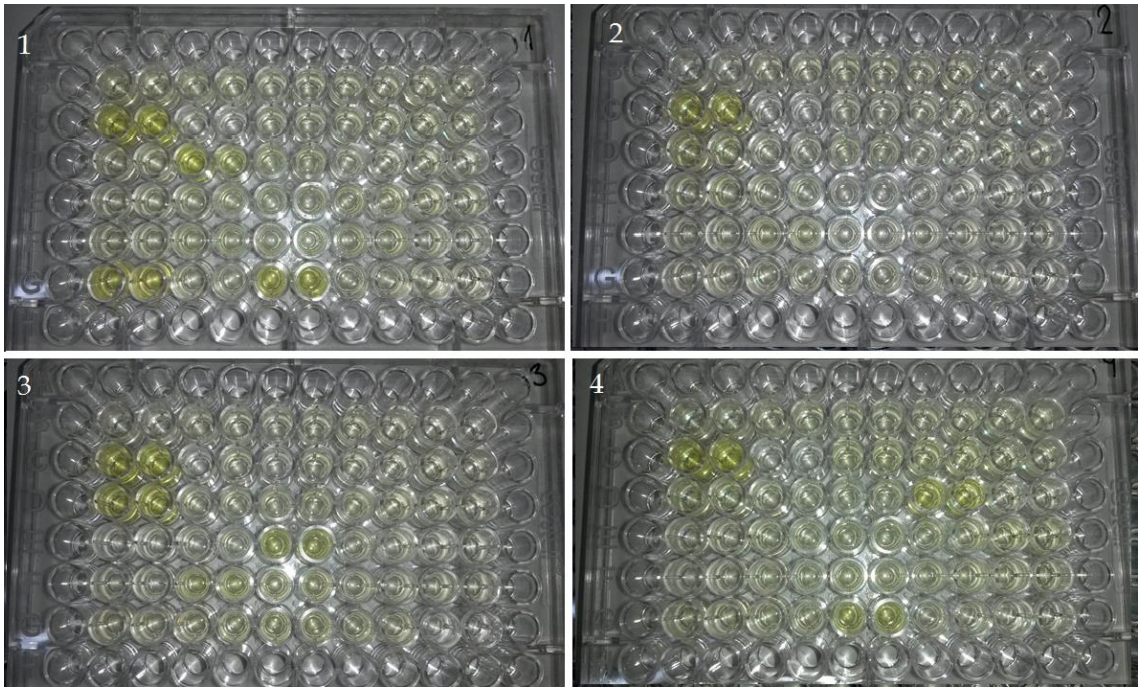
WMV-plates (5-8) from farms #1 - #11



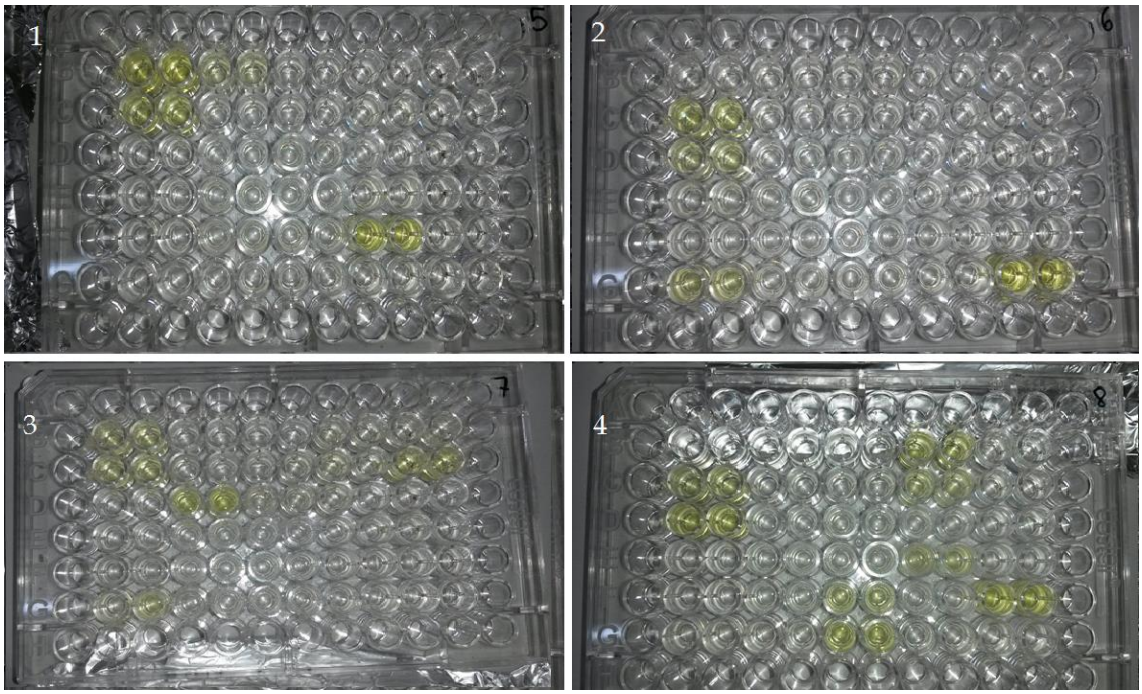
CMV-Plates (9-12) from farms #1 - #11



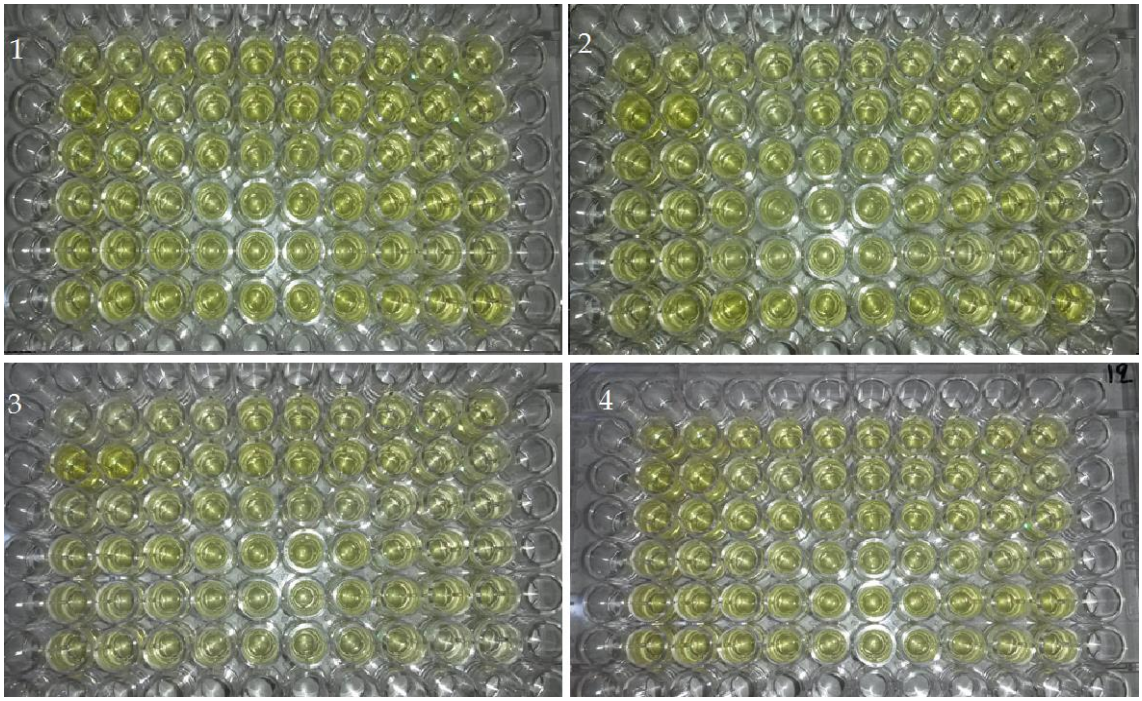
CMV-Plates (1-4) from farms #21 – #30



ZYMV-Plates (5-8) from farm #21 – #30



WMV-plates (9-12) from farms #21 - #30



ZYMV-plates 1-4 from farms #1 - #11 ELISA-reader results after 30 minutes.

0,177	0,158	0,149	0,178	0,210	0,186	0,176	0,181	0,183	0,158
0,205	0,207	0,215	0,175	0,189	0,246	0,216	0,200	0,315	0,191
0,138	0,168	0,208	0,185	0,186	0,222	0,118	0,140	0,156	0,144
0,175	0,168	0,244	0,184	0,190	0,234	0,192	0,187	0,208	0,173
0,146	0,152	0,136	0,140	0,156	0,149	0,153	0,160	0,150	0,137
0,197	0,208	0,259	0,276	0,173	0,161	0,210	0,178	0,171	0,161

0,207	0,189	0,244	0,254	0,184	0,203	0,251	0,237	0,188	0,184
0,235	0,255	0,177	0,187	0,163	0,211	0,184	0,166	0,189	0,219
0,279	0,200	0,188	0,178	0,147	0,152	0,196	0,205	0,172	0,185
0,307	0,362	0,176	0,170	0,164	0,177	0,175	0,187	0,171	0,191
0,270	0,248	0,127	0,143	0,135	0,135	0,142	0,136	0,145	0,172
0,195	0,251	0,236	0,171	0,175	0,181	0,173	0,176	0,202	0,228

0,147	0,143	0,155	0,144	0,154	0,147	0,187	0,197	0,154	0,150
0,158	0,168	0,131	0,133	0,199	0,130	0,135	0,195	0,137	0,155
0,161	0,162	0,204	0,299	0,157	0,185	0,592	0,168	0,170	0,167
0,177	0,215	0,204	0,229	0,176	0,170	0,171	0,211	0,220	0,210
0,183	0,134	0,145	0,267	0,150	0,202	0,181	0,179	0,239	0,199
0,166	0,203	0,205	0,183	0,196	0,184	0,194	0,187	0,167	0,172

0,142	0,204	0,892	0,849	0,172	0,208	0,230	0,171	0,187	0,212
0,241	0,266	0,189	0,178	0,209	0,193	0,225	0,145	0,136	0,146
0,198	0,203	0,184	0,166	0,214	0,148	0,154	0,235	0,136	0,273
0,272	0,290	0,225	0,205	0,251	0,276	0,219	0,276	0,204	0,259
0,229	0,216	0,236	0,277	0,280	0,321	0,302	0,426	0,163	0,161
0,384	0,250	0,263	0,278	0,307	0,200	0,166	0,184	0,163	0,164

WMV-plates 5-8 from farms #1 - #11 ELISA-reader results after 30 minutes.

0,190	0,174	0,150	0,146	0,163	0,138	0,148	0,148	0,155	0,170
0,233	0,233	0,158	0,155	0,216	0,225	0,147	0,199	0,204	0,261
0,174	0,168	0,186	0,171	0,170	0,164	0,189	0,277	0,230	0,295
0,177	0,199	0,194	0,218	0,200	0,171	0,200	0,188	0,177	0,162
0,176	0,155	0,153	0,164	0,169	0,168	0,176	0,191	0,220	0,372
0,180	0,168	0,189	0,174	0,152	0,158	0,153	0,160	0,155	0,180

0,182	0,168	0,299	0,202	0,227	0,172	0,171	0,182	0,177	0,187
0,354	0,264	0,167	0,142	0,165	0,175	0,175	0,178	0,216	0,209
0,146	0,200	0,165	0,160	0,181	0,172	0,158	0,163	0,185	0,275
0,185	0,253	0,190	0,179	0,174	0,177	0,260	0,301	0,188	0,194
0,169	0,264	0,157	0,141	0,158	0,162	0,151	0,151	0,174	0,157
0,270	0,230	0,182	0,173	0,169	0,177	0,171	0,174	0,202	0,223

0,193	0,162	0,178	0,195	0,239	0,210	0,216	0,255	0,258	0,207
0,285	0,243	0,162	0,190	0,224	0,264	0,237	0,243	0,192	0,230
0,232	0,251	0,264	0,298	0,202	0,215	0,141	0,138	0,181	0,210
0,271	0,270	0,212	0,234	0,244	0,268	0,213	0,264	0,294	0,317
0,221	0,217	0,193	0,220	0,183	0,212	0,186	0,262	0,236	0,246
0,223	0,203	0,267	0,210	0,172	0,211	0,183	0,181	0,181	0,234

0,149	0,160	0,180	0,193	0,155	0,162	0,160	0,157	0,193	0,206
0,243	0,247	0,169	0,145	0,155	0,227	0,204	0,163	0,175	0,193
0,161	0,195	0,217	0,201	0,164	0,225	0,121	0,145	0,158	0,157
0,256	0,221	0,215	0,190	0,165	0,222	0,192	0,197	0,189	0,219
0,242	0,222	0,153	0,159	0,178	0,154	0,163	0,163	0,200	0,152
0,184	0,204	0,171	0,224	0,182	0,210	0,157	0,165	0,136	0,155

CMV-plates 9-12 from farms #1 - #11 ELISA-reader results after 30 minutes.

0,365	0,329	0,297	0,304	0,382	0,425	0,282	0,291	0,330	0,368
0,577	0,688	0,192	0,162	0,393	0,396	0,295	0,293	0,344	0,355
0,306	0,293	0,261	0,257	0,226	0,243	0,275	0,273	0,307	0,284
0,261	0,263	0,261	0,250	0,283	0,271	0,255	0,248	0,310	0,414
0,251	0,211	0,236	0,229	0,281	0,320	0,257	0,267	0,258	0,252
0,294	0,318	0,258	0,251	0,419	0,468	0,449	0,408	0,304	0,301

0,293	0,270	0,355	0,340	0,343	0,358	0,357	0,399	0,261	0,257
0,582	0,689	0,239	0,199	0,468	0,337	0,545	0,384	0,261	0,296
0,319	0,363	0,361	0,331	0,289	0,331	0,334	0,319	0,226	0,187
0,296	0,280	0,295	0,289	0,302	0,313	0,475	0,438	0,435	0,428
0,402	0,365	0,290	0,271	0,436	0,523	0,356	0,412	0,484	0,522
0,323	0,320	0,368	0,310	0,283	0,318	0,342	0,340	0,339	0,309

0,286	0,270	0,282	0,274	0,362	0,362	0,417	0,337	0,331	0,253
0,528	0,590	0,208	0,141	0,206	0,210	0,309	0,213	0,213	0,236
0,254	0,249	0,257	0,282	0,217	0,196	0,160	0,196	0,163	0,200
0,452	0,515	0,236	0,231	0,388	0,380	0,213	0,222	0,260	0,309
0,235	0,252	0,258	0,252	0,165	0,174	0,241	0,277	0,225	0,222
0,242	0,228	0,270	0,182	0,151	0,181	0,167	0,202	0,244	0,273

0,263	0,219	0,291	0,292	0,201	0,184	0,255	0,208	0,195	0,222
0,600	0,649	0,142	0,142	0,171	0,182	0,190	0,199	0,175	0,217
0,232	0,172	0,203	0,246	0,156	0,189	0,169	0,170	0,132	0,197
0,270	0,328	0,272	0,246	0,222	0,204	0,188	0,186	0,241	0,231
0,214	0,191	0,183	0,170	0,187	0,192	0,244	0,182	0,154	0,204
0,298	0,230	0,275	0,216	0,210	0,279	0,223	0,226	0,220	0,225

CMV-plates 1-4 from farms #21 - #30 ELISA-reader results after 30 minutes.

0,359	0,345	0,270	0,326	0,338	0,294	0,222	0,264	0,235	0,243
1,258	1,154	0,204	0,152	0,304	0,282	0,222	0,240	0,228	0,261
0,280	0,330	0,984	0,562	0,319	0,258	0,210	0,209	0,211	0,238
0,313	0,336	0,311	0,276	0,314	0,222	0,245	0,245	0,252	0,311
0,285	0,212	0,274	0,212	0,524	0,179	0,177	0,184	0,168	0,190
0,938	0,743	0,297	0,228	0,756	0,735	0,212	0,228	0,218	0,205

0,185	0,177	0,232	0,290	0,253	0,237	0,277	0,276	0,198	0,217
0,848	0,843	0,128	0,146	0,221	0,240	0,235	0,228	0,162	0,171
0,409	0,331	0,222	0,215	0,216	0,229	0,207	0,241	0,191	0,219
0,236	0,262	0,212	0,206	0,248	0,188	0,202	0,183	0,201	0,197
0,222	0,201	0,290	0,310	0,161	0,166	0,208	0,221	0,216	0,201
0,237	0,219	0,217	0,218	0,215	0,220	0,200	0,206	0,214	0,185

0,178	0,177	0,249	0,238	0,236	0,228	0,261	0,261	0,222	0,183
0,977	0,929	0,136	0,227	0,146	0,269	0,214	0,222	0,221	0,197
0,782	0,772	0,204	0,215	0,218	0,233	0,317	0,276	0,284	0,239
0,212	0,192	0,188	0,192	0,533	0,576	0,236	0,299	0,259	0,237
0,216	0,167	0,434	0,390	0,273	0,310	0,239	0,232	0,238	0,257
0,305	0,268	0,275	0,285	0,352	0,339	0,284	0,333	0,183	0,178

0,190	0,288	0,238	0,290	0,257	0,241	0,224	0,682	0,251	0,378
0,959	1,116	0,216	0,144	0,305	0,345	0,345	0,336	0,241	0,239
0,327	0,255	0,247	0,235	0,206	0,224	0,688	0,656	0,190	0,199
0,222	0,211	0,230	0,232	0,225	0,224	0,215	0,227	0,332	0,338
0,180	0,165	0,240	0,196	0,249	0,281	0,215	0,218	0,246	0,245
0,242	0,239	0,257	0,244	0,495	0,502	0,171	0,173	0,166	0,176

ZYMV-plates 5-8 from farms #21 - #30 ELISA-reader results after 30 minutes.

1,082	1,144	0,319	0,320	0,115	0,119	0,115	0,114	0,114	0,107
0,686	0,698	0,110	0,110	0,108	0,111	0,115	0,119	0,115	0,119
0,116	0,113	0,114	0,110	0,114	0,118	0,117	0,117	0,112	0,118
0,144	0,149	0,215	0,146	0,129	0,132	0,134	0,142	0,138	0,133
0,162	0,105	0,130	0,128	0,103	0,110	0,830	0,821	0,103	0,105
0,211	0,180	0,149	0,136	0,131	0,146	0,135	0,161	0,132	0,129

0,111	0,113	0,118	0,207	0,122	0,118	0,129	0,202	0,136	0,217
0,659	0,604	0,126	0,117	0,116	0,158	0,120	0,170	0,175	0,147
0,442	0,473	0,121	0,118	0,116	0,108	0,107	0,123	0,114	0,114
0,140	0,193	0,222	0,140	0,167	0,123	0,140	0,135	0,125	0,132
0,152	0,152	0,097	0,118	0,114	0,099	0,114	0,104	0,103	0,110
0,552	0,550	0,215	0,163	0,186	0,143	0,146	0,157	1,232	1,234

0,447	0,490	0,111	0,110	0,123	0,187	0,147	0,157	0,162	0,210
0,436	0,478	0,153	0,105	0,125	0,256	0,270	0,137	0,561	0,694
0,129	0,113	0,644	0,795	0,135	0,118	0,138	0,536	0,153	0,119
0,155	0,236	0,169	0,134	0,126	0,124	0,127	0,126	0,178	0,140
0,131	0,133	0,118	0,099	0,098	0,096	0,100	0,101	0,162	0,145
0,146	0,188	0,191	0,166	0,135	0,121	0,141	0,136	0,119	0,116

0,107	0,109	0,115	0,105	0,109	0,115	0,598	0,619	0,225	0,119
0,473	0,465	0,113	0,108	0,111	0,117	0,506	0,733	0,279	0,122
0,773	0,693	0,109	0,107	0,121	0,137	0,196	0,248	0,392	0,232
0,128	0,138	0,144	0,124	0,130	0,178	0,865	0,543	0,302	0,522
0,108	0,110	0,108	0,120	0,408	0,384	0,279	0,212	0,955	0,935
0,218	0,200	0,168	0,147	0,660	0,653	0,132	0,239	0,287	0,435

WMV-plates 9-12 from farms #21 - #30 ELISA-reader results after 30 minutes.

0,432	0,428	0,581	0,630	0,688	0,588	0,533	0,572	0,526	0,521
0,971	0,975	0,394	0,426	0,603	0,664	0,668	0,575	0,562	0,560
0,550	0,456	0,430	0,458	0,406	0,576	0,432	0,506	0,421	0,364
0,471	0,472	0,358	0,384	0,452	0,522	0,571	0,545	0,633	0,555
0,496	0,399	0,497	0,423	0,434	0,363	0,377	0,428	0,318	0,408
0,532	0,598	0,606	0,631	0,623	0,579	0,617	0,627	0,554	0,744

0,426	0,392	0,385	0,378	0,346	0,343	0,349	0,335	0,375	0,453
0,865	0,835	0,343	0,289	0,461	0,423	0,407	0,431	0,381	0,388
0,480	0,413	0,486	0,462	0,365	0,399	0,375	0,331	0,341	0,325
0,349	0,377	0,382	0,574	0,364	0,425	0,427	0,445	0,496	0,390
0,357	0,358	0,371	0,287	0,342	0,357	0,290	0,271	0,317	0,329
0,345	0,469	0,740	0,482	0,511	0,498	0,559	0,416	0,436	0,676

0,385	0,497	0,263	0,298	0,424	0,413	0,341	0,348	0,360	0,439
1,254	1,148	0,412	0,379	0,497	0,490	0,444	0,412	0,286	0,279
0,301	0,295	0,333	0,406	0,328	0,291	0,244	0,227	0,244	0,300
0,372	0,419	0,477	0,394	0,392	0,420	0,425	0,354	0,326	0,335
0,359	0,247	0,300	0,297	0,300	0,260	0,355	0,337	0,328	0,323
0,368	0,321	0,318	0,288	0,304	0,292	0,295	0,298	0,207	0,206

0,672	0,634	0,565	0,550	0,541	0,506	0,563	0,492	0,555	0,536
0,927	0,815	0,407	0,358	0,466	0,413	0,372	0,462	0,586	0,353
0,676	0,617	0,528	0,482	0,506	0,509	0,506	0,462	0,422	0,509
0,500	0,472	0,410	0,420	0,422	0,416	0,396	0,386	0,445	0,550
0,575	0,520	0,471	0,503	0,536	0,536	0,488	0,509	0,564	0,803
0,592	0,508	0,475	0,511	0,420	0,412	0,396	0,444	0,394	0,441