

## Serological detection of close and distant relationships of an isolated *Tobamovirus*

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### Abstract

**A virus causing mosaic and leaf yellowing symptoms on *Mucuna pruriens* was isolated and purified through its susceptible host *Vigna unguiculata* var. TVu 76. Yield of the purified virus obtained was 7.2 mg/kg. It had an ultraviolet (UV) absorption spectrum at 260 nm as  $1.53 \pm 0.00$  and at 280 nm as  $1.45 \pm 0.00$ , which translated into nucleic acid content of 72.9 %. Determination of the molecular weight of the virus coat protein from a plotted graph gave 27 kiloDaltons. When the purified virus was electrophoresed in agarose gel, no secondary band was observed. Absorbance curve at 260 nm was obtained when the purified virus was measured spectrophotometrically. A volume of 16 000 $\mu$ L antiserum was obtained against the virus with concentration of 0.3 mg/ml. The titre value determined for the antiserum raised against the virus was 1:2000 as there was a wider difference between the readings obtained for the healthy and diseased controls. At this dilution, the titre value was two and a half times greater than that of the healthy control. The heterologous: homologous percentage (%) obtained for the isolated virus and TMV (Muguga strain) was 50.0 % when Protein-A sandwich enzyme linked immunosorbent assay (PAS ELISA) was used to determine its serological relationship with the virus and other *Tobamovirus* strains.**

**Keyword:** PAS ELISA, Tobamovirus, coat protein, molecular weight

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### Introduction

Enzyme linked immunosorbent assay described by Clark and Adams (1977) showed that the microplate method of ELISA could be very effectively applied to the detection and assay of plant viruses (Matthew, 1991). Since that time, the method has been more widely used (Matthews, 1991). The method is a serological procedure and has been used to detect various viruses in yams (*Dioscorca alata*) (Odu et al., 1999; Eni et al., 2008), legumes (Odedara et al., 2007; Odedara et al., 2012), *Solanaceous* plants, cucurbits (Ayo-John et al.,

2014) and Citrus (Kareem et al., 2014).

Protein A-sandwich (PAS) ELISA is an indirect ELISA (Hughes & Thomas, 1988) and it makes use of protein-A, a molecule isolated from the cell wall of *Staphylococcus aureus*. The binding of Protein-A is used in several serological procedures (Matthews, 1991). For instance, an indirect Protein-A sandwich (PAS-ELISA) which was more successful than double antibody sandwich (DAS) ELISA in detecting heterologous isolates of cherry leaf roll virus was described by Edwards & Cooper (1985). It was hypothesized that a het:hom PAS ELISA could be used to

detect both close and distant serological relationships of *Tobamovirus* (Hughes and Thomas, 1988).

Other viruses belonging to this group are: *Tomato mosaic virus* (ToMV), *Pepper mild mottle virus* (PMMV), *Cucumber green mottle mosaic virus* (CGMMV), *Sunnehemp mosaic virus* (SHMV), *Ullucus mild mottle virus* (UMMV) (Hughes & Thomas, 1988). They are rod-shaped rigid tube viruses. Hosts for TMV include tobacco, tomato and other *Solanaceous* plants. TMV had caused 20 % loss on tomato (Scholthof, 2000).

Growth of *Mucuna pruriens* had been emphasized for enhancement of soil fertility, prevention of soil erosion and maintenance of soil physical structures (Versteeg & Koudokpon, 1993, Sanginga et al., 1996; Makau & Gachene, 2001). Viral symptoms expression such as mosaic, leaf yellowing and leaf distortion sometimes accompanying growth of *M. pruriens*. Some previous reports had shown that this plant is sometimes infected with TMV. Hence, the present study was conducted with the following objectives: to purify the *Tobamovirus* from the naturally infected host, determine the molecular weight of its coat protein, analyze its serological relationships with its other strains and produce antibodies against the virus.

## Materials and Methods

### *Isolation of the Tobamovirus and Purification*

Virus from *Mucuna pruriens* was propagated in cowpea (*Vigna unguiculata* var TVu 76) by homogenizing symptomatic leaves of *Mucuna pruriens* in sterilized mortars and pestles in 0.1M phosphate buffer (pH 7.7) containing 0.01M ethylene diaminetetraacetic acid and rubbing the extracted sap on carborundum dusted leaves of a week old cowpea seedlings (TVu 76). At 2 weeks after inoculation 70 g of systemically infected cowpea leaves were harvested and homogenized in a waring laboratory blender (Model No 35-59, Christison, USA) with 0.5M phosphate buffer (pH 7.2) containing 1% 2-mercapto ethanol in the rate of 1g tissue to 2ml buffer. After straining the homogenate through cheesecloth, 8ml N-butanol was added to every 100ml of extract while stirring for further 15 min before

centrifugation at 9,500 rpm for 30 min. The supernatant was discarded into a clean beaker and 4.0 g polyethylene glycol (PEG mol. wt. 8000) was added for every 100 ml of the virus suspension. This was stirred for 1h at 4°C. Centrifugation was done at 10,000 g for 15 min and the pellet resuspended in 20 ml 0.01M phosphate buffer (pH 7.2) (Gooding & Herbert, 1967).

To obtain a pure virus suspension, 0.4 g sodium chloride (NaCl) and 0.4 g PEG were added for every 10 ml of the suspension while stirring and this continued till the salt dissolved and the suspension centrifuged at 9,500 rpm for 15 minutes. The supernatant was discarded and the pellets resuspended in 2 ml 0.01M phosphate buffer for each 100 ml of the initial extract. Centrifugation was done for the suspension at 10,000 g for 5 minutes in order to remove the contaminants (Gooding and Herbert, 1967). The absorbance of the virus at 260 and 280 nm were estimated and virus stored in 0.01M phosphate buffer (pH 7.2) at -20°C from where it was withdrawn for antiserum production and other tests.

### *Spectrophotometry of purified preparations of isolated Tobamovirus.*

The absorption spectrum of the purified preparations of the isolated *Tobamovirus* was determined with a Beckman DU 520 Model Spectrophotometer (Beckman Instruments, USA) in order to ascertain the purity of the preparation and to obtain information on concentration and the nucleic acid content of the virus (Hughes, 1986). A silica quartz cuvette (Sigma, USA) containing 1 ml of resuspending buffer solution was placed in the cuvette chamber with the blank side positioned in the path of the light beam and the scale set to zero absorbance (100% transmittance) at 260 and 280 nm. The absorbance of the cuvette containing the virus preparation was also taken at 260 and 280 nm after the resuspending buffer has been discarded from the cuvette. The ratio of the absorbance of the virus preparations at 260 and 280 nm was then used to estimate the amount of nucleic acid in the preparation using the following formula (Gibbs & Harrison, 1976):

$$A_{260}/A_{280} = 0.932 + 0.045 (\text{RNA}\%) - 0.0006 (\text{RNA}\%)^2$$

Concentration of the purified virus was calculated based on the extinction co-efficient of the virus from the formula described by Hughes (1986).

$$C = \frac{OD_{260} \times D \times 1000}{E}$$

where;

C = concentration of the virus in mg/ml; OD = optical density at 260 nm; D = dilution of the virus preparations; E = Extinction co-efficient of the virus

The specific extinction coefficient of the virus used was as described by Hollings & Brunt, (1981). It could also be estimated by substituting the estimated percentage RNA in the following equation according to Gibbs & Harrison, (1976).

$$(E^{0.1\%}_{1\text{cm}, 260\text{nm}}) = 1.531 + 2.05 (\text{RNA}\%)$$

The specific extinction coefficient of a virus is the optical density of 1 cm layer of 1mg/ml (0.1%) virus preparation at a wavelength of 260 nm. From the former formula, the concentration of the virus, was then adjusted to 1mg/ml by using a normal saline solution (0.95 % NaCl) in sterile distilled water to dilute out the purified virus which was kept in Eppendorf tubes and stored at -20°C from where each was withdrawn for rabbit injection in the production of polyclonal antibody. To find the yield of the virus from a given weight of plant tissue, the formula (Hughes, 1986) was used as below.

$$Y = \frac{C \times V \times 1000}{W}$$

where;

Y = yield of virus (mg/kg fresh weight); V = volume of the purified virus (ul); C = concentration of the virus (mg/ml); W = fresh weight of plant tissue (kg)

#### *Production of antiserum against the isolated Tobamovirus.*

Partially purified virus from *M. pruriens* at a concentration of 1mg/ml mixed with equal volume of Freund's complete adjuvant was injected intramuscularly into a 3-4 months old rabbit (Hampton et al., 1990). Healthy blood was collected from the rabbit before injection to serve as a control during serological analysis. Two weeks after the first injection, 1 ml of the virus, at concentration of 1 mg/ml mixed with Freund's incomplete adjuvant was injected

intramuscularly into the second rear thigh of the rabbit. The third and fourth injections were given as previously described for second injection. Fourth injection, which was the booster injection was done 4 weeks after the third injection. Blood was collected after the last injection from the ear vein of the rabbit caged in a rabbit cage (Nalgene, Italy, Model No COD 16 0012).

The tip of one of the ear was shaved with new razor blade, a cotton wool soaked with xylene was placed just at the edge of the ear opposite the place where a cut was made to allow vein dilation. The shaved area was cleansed using a cotton wool soaked with absolute ethanol and an ear vein that runs through the area was cut with a clear razor blade. Drops of blood were collected into a clean small sized beaker (PYREX, USA). Serum was obtained from the blood by incubating the blood at 37°C for 6 h during which the clot was ringed with a needle to allow shrinkage (Hughes, 1986). The serum was collected at 6,000 rpm for 10 minutes and an equal volume of glycerol added before storing at 4°C.

#### *Titre value determination for the antiserum produced against the isolated Tobamovirus.*

The titre of the antiserum produced against the isolated *Tobamovirus* was determined using the PAS-ELISA described by Hughes (1986). A two-fold serial dilutions of antiserum to the isolated *Tobamovirus* was made in phosphate buffer saline with Tween 20 PBS-T-containing 0.15M NaCl, 0.003M KCl, pH 7.4 with 0.05% tween 20). For a particular dilution of an antiserum, two duplicate wells were used for both the infected sap (antigen) and the healthy (negative control) sap. During the addition of antibodies, wells in the same row were coated with different dilutions of crude antibody while duplicated wells were coated with similar dilutions of antisera. Description of protein-A sandwich ELISA wells of microtitre plates were precoated with protein-A at a concentration of 1 ug/ml in 0.05M Na<sub>2</sub>CO<sub>3</sub> coating buffer, pH 9.6.

Plates were incubated at 37°C for 2 h after which they were emptied and washed by flooding the wells with 0.02M phosphate buffered saline containing 0.015M NaCl and 0.003M KCl, pH 7.4 with 0.05% tween -20 (PBS-T).

After each wash, plates were allowed to stay for 3 min before the wells were emptied. Plates were drained and tapped dry after two more washings. The corresponding antiserum against the isolated *Tobamovirus* that have been serially diluted appropriately in PBS-T were added to the wells as 100 ul/well and plates incubated at 37°C for 2 h, washed and dried as earlier described. Infected leaf samples of TVu 76 containing the isolated *Tobamovirus* were ground as 0.1g/ml in PBS-T containing 2% w/v poly vinyl pyrrolidone (PVP) and incubated as 100 ul/well overnight at 4 °C. Leaf extract of healthy TVu 76 were coated into the first duplicate wells of the inner sixty wells of the ELISA plate. Outer wells were not used to prevent errors. The third duplicate wells were coated with sap of the infected TVu 76.

Plates were washed as above and the serially diluted antiserum in PBS-T was added appropriately to each well as 100ul/well well and then incubated at 37 °C for 2 h before washing and drying. Plates were developed by addition of 200 ul aliquots/well of enzyme substrate (para-nitro phenyl phosphate) dissolved at a concentration of 1mg/ml in substrate buffer which consisted of 12% diethanolamine adjusted with concentrated HCl to pH 9.8. Incubation was done at room temperature for 1 hr and absorbance readings taken at 405 nm with Dynex MRX ELISA reader (UK).

Absorbance values obtained for each serially diluted antiserum were plotted against those values for healthy controls and the titre determined from the graph.

#### *Determination of serological relationship between the isolated Tobamovirus and other Tobamoviruses.*

Serological relationship between virus isolated from *Mucuna pruriens* and other viruses to which antisera were available was carried out by challenging each of the 5 antisera available for these *Tobamoviruses* against the virus isolated from *Mucuna pruriens* in extracted sap of infected Cowpea (*Vigna unguiculata* var. TVu 76) according Hughes and Thomas (1988).

Two wells of Dynatech microtitre plate were filled per antiserum for the infected sap and other two wells which served as control were

filled with PBS-T. The homologous antiserum produced against the isolated Tobamovirus from *M. pruriens* was diluted in PBS-T as 1:1000 respectively and filled into the four wells per each antiserum used to detect the virus isolate.

The mean absorbance value at 405 nm ( $A_{405}$  nm) obtained for each control was subtracted from the mean value obtained for virus-containing wells. Corrected values were used to calculate heterologous: homologous (het:hom) ratio expressed in percentage using the formula (Hughes & Thomas, 1988) as below:

$$\frac{Het:hom}{Hom:hom} \times 100 = het: hom\%$$

#### *Determination of Molecular weight of the coat protein of the isolated Tobamovirus*

Denatured, purified preparations of the isolates were used to determine the molecular weight of the isolated *Tobamovirus*, Discontinuous, denaturing polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to Laemmli (1970). The denatured virus isolate with the extracted protein from healthy (negative control) *Vigna unguiculata* var. TVu 76 were subjected to this process. The molecular weight of the viral coat protein was estimated from the plotted graph of logarithms of molecular weights of standard protein markers against their Rf values (ratio of the distance migrated by individual protein to total distance migrated by the tracking dye from the base line of the stacking gel).

## **Results**

The purified preparations of the isolated *Tobamovirus* appeared milky in the resuspension buffer. The volume of the virus obtained was 1000 ul The yield when calculated was 7.2 mg/kg of fresh leaves of *Vigna unguiculata* var. TVu 76 (Table 1). The ultraviolet absorption spectrum of the purified isolated virus had a minimum absorbance at 260 nm of  $1.53 \pm 0.00$  while at 280 nm it was  $1.45 \pm 0.00$ . This translated to a nucleic acid content of 72.9%. The antiserum raised against the isolated *Tobamovirus* from *Mucuna pruriens* was 16,000 ul in volume with concentration of 0.3 mg/mL. An ultraviolet absorption spectrum curve was obtained at 260 nm for the purified isolated *Tobamovirus* (Fig 1).

Table 1: Yield parameters of the purified isolated Tobamovirus preparation

Parameter measured	Value
Mass of leaf tissue (g)	70.1
Volume of virus preparation ( $\mu$ l)	10,000
Absorbance at 260 nm ( $A_{260\text{nm}}$ )	1.53 $\pm$ 0.00
Absorbance at 280 nm ( $A_{280\text{nm}}$ )	1.45 $\pm$ 0.00
Yield (mg/kg)	7.2
Virus concentration ( $\mu$ g/ml)	5,100
$A_{260\text{nm}}/A_{280\text{nm}}$	1.05

During electrophoresis of the purified virus on the gel, a clear band was obtained for the purified virus in the undiluted form (Fig 2). There were no secondary bands. When the molecular weight of the coat protein of the virus was determined from the plotted graph, a molecular weight of 27 kiloDalton was obtained (Fig. 3).

At 1:250 dilution, the antiserum when reacted against the virus-infected sap of *V. unguiculata* var. TVu 76, gave absorbance values that were four times greater than that of the healthy control (Fig. 4). Between 1:1000 and 1:4000 dilutions, the titre values were more or less stable, being about two and half times greater than that of the healthy control. Therefore, a working dilution of 1:2000 was recommended

for use.

The reaction of isolated *Tobamovirus* to antiserum against TMV (Muguga), gave a strong immunogenic reaction. This showed that they were closely related with the het:hom % relationship of 50.0 (Table 2). The reactions of the isolated *Tobamovirus* to other antisera raised against other Tobamoviruses such as *Tomato Mosaic virus* (ToMV) genus *Tobamovirus*, *Ullucus mild mottle virus* genus *Tobamovirus* and *Pepper mild mottle virus* genus *Tobamovirus* were weakly immunogenic, thus suggesting that they are distantly related. The het:hom % relationships were 39.9 %; 35.6 % and 28.7 % respectively (Table 2).

Table 2: Serological relationship of the isolated Tobamovirus to other strain

Antiserum against:	Source	Mean of $A_{405\text{nm}}$ for sample	Mean of $A_{405\text{nm}}$ for PBS-T control wells	Heterologous: homologous (%)
Isolated Tobamovirus	IITA	0.483	0.160	100.0
Tobacco mosaic virus genus Tobamovirus (Muguga)	A.A. Brunt, UK	0.330	0.169	50.0
Tobacco mild green mosaic virus genus Tobamovirus	A.A. Brunt, UK	0.292	0.146	45.0
Tomato mosaic virus genus Tobamovirus	A.A. Brunt, UK	0.275	0.146	39.9
Ullucus mild mottle virus genus Tobamovirus	A.A. Brunt, UK	0.268	0.153	35.6
Pepper mild mottle virus genus Tobamovirus	A.A. Brunt, UK	0.268	0.175	28.7

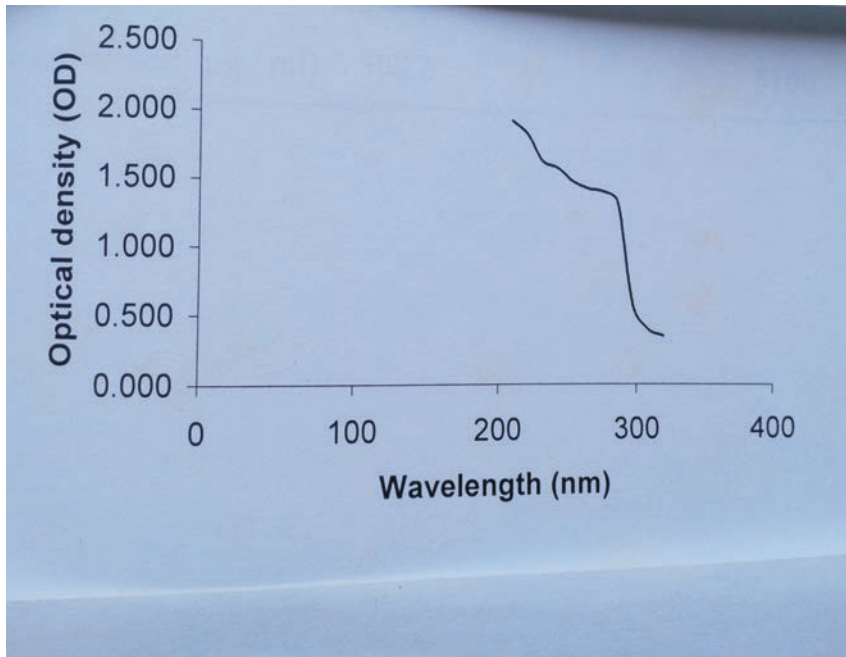


Fig. 1: Absorption spectrum of the purified isolated Tobamovirus.

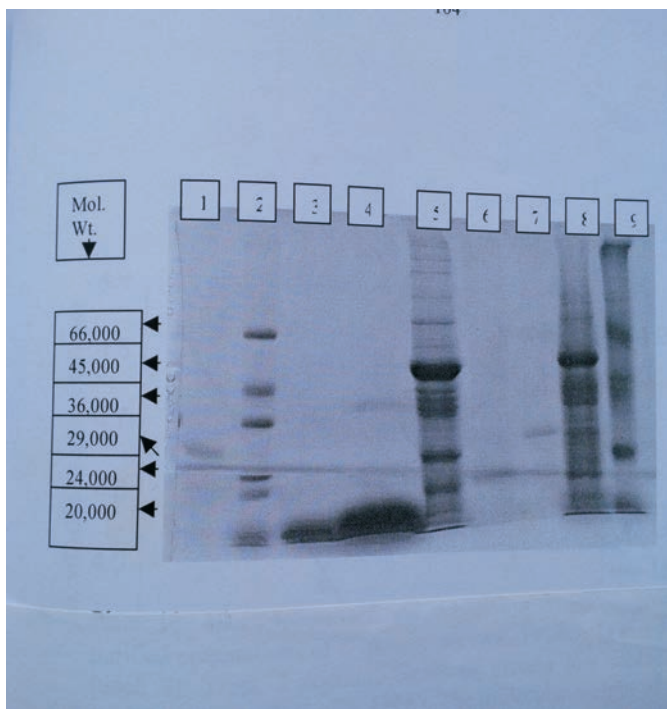


Fig 2: Electrophoresed gel containing migrated coat protein of the purified preparation of the isolated Tobamovirus

Keys: Track 2 contains band of protein low molecular weight range markers (Sigma, St LOIUS, USA). Molecular weights of the markers are in kiloDaltons (kDa). Track 3: Purified preparation of the isolated Tobamovirus in diluted form (1:10). Track 4: Purified preparation of the isolated Tobamovirus undiluted). Track 5: Clarified sap of healthy cowpea (*Vigna unguiculata* var. TVu 76)

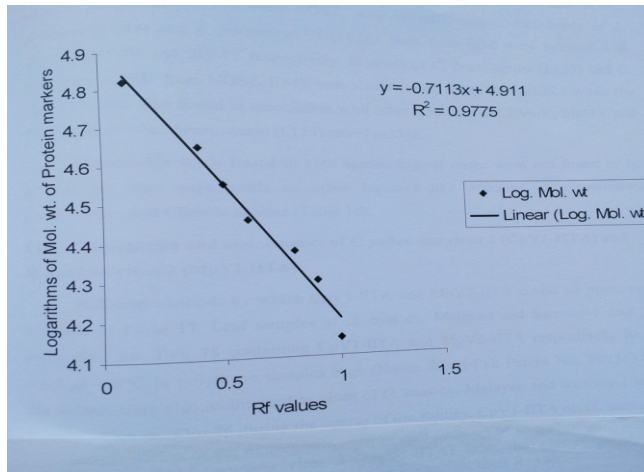


Fig.3: Determination of the molecular weight of coat protein of the isolated Tobamovirus in purified form from the plotted graph of logarithm of molecular weight of protein markers against their Rf values.

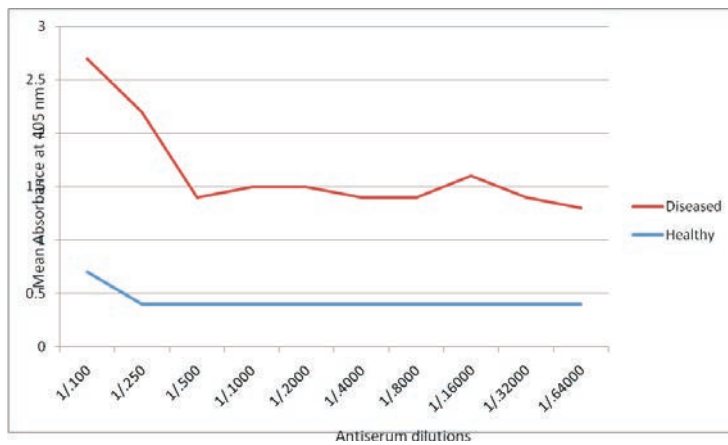


Fig.4: Mean absorbance values of antiserum against the isolated Tobamovirus at 405 nm during different dilutions

Antiserum against Tobacco mild green virus genus *Tobamovirus* however reacted moderately with the isolated *Tobamovirus* with het:hom % of 45.0. This showed that Tobacco mild green virus and the isolated *Tobamovirus* from *M. pruriens* were related to some extent but not closely.

**Discussion**

A *Tobamovirus* was isolated from naturally infected *Mucuna pruriens* collected from the campus of International Institute of Tropical Agriculture (IITA), Ibadan. Symptoms observed on the plant include mosaic, yellowing and leaf distortion.

Purification of this virus was carried out and it was observed that there was a high yield of the purified virus as observed in the milky appearance of the preparation and the

presence of a curve at 260 nm when absorbance value of the purified preparation was measured spectrophotometrically. This might be due to many factors ranging from the host species to the conditions of the extraction medium used during purification procedure. As Matthew (1991) stated that some inhibitory substances such as phenolic materials, organic acids, mucilage, gums, certain proteins and enzymes particularly ribonucleases do inhibit the isolation of some viruses from their natural hosts. Examples are plant members of the *Rosaceae* which contain high concentration of tannins in their leaves. For the isolated *Tobamovirus* to have a high yield as obtained in this study means that the host on which this virus was maintained did not possess such inhibitory substances.

The ultra violet absorption spectrum curve for the isolated *Tobamovirus* at 260 nm

showed that there was viral protein in the preparation as absence of curve means no virus protein in the preparation. In addition, ultra violet absorption spectrum gives information on the possible presence of impurities in the virus preparation as the wide deviation from 260 nm signifies presence of impurities. But the curve of the isolated *Tobamovirus* did not deviate from 260 nm signifying the absence of impurities.

The production of large quantities of serum by the isolated virus must have been due to its high immunogenicity. Members of the *Tobamovirus* group had been described by Gooding and Herbert (1967) as good immunogen that triggered the production of good volume of antibodies. Other factor responsible for the immunogenicity of an antigen was molecular weight of an antigen. Large antigen molecules are usually more effective than small ones (Matthew, 1991). Size of an animal in which the antiserum was produced is also another factor that determines the amount of antiserum produced. Since the antiserum to the *Tobamovirus* was raised in a Rabbit, a useful volume of antiserum was obtained when compared to the volume that would have been obtained if a mouse was used (Hampton et al., 1990).

When the homologous (hom) antiserum was used against the isolated *Tobamovirus* to trap the virus and different heterologous (het) antibodies, there were diverse reactions. The het:hom % relationship obtained for TMV (Muguga strain) was 50.0 %. This showed that they were closely related. This is in accordance with the study of Hughes and Thomas (1988) who also detected a similar het:hom % value between *Tomato mosaic virus* genus *Tobamovirus* (Aucuba isolate, ToMV-A) and *Tomato mosaic virus* (strain Z, ToMV-Z). The relationship was able to exist because the isolated *Tobamovirus* must have bound very strongly with the antiserum of TMV (Muguga strain) due to high affinities between the two.

The reactions of the isolated TMV from *M. pruriens* to other antisera against *Tomato mosaic virus*, *Ullucus mild mottle virus* and *Pepper mild mottle virus* (PMMV) were weakly immunogenic with het:hom values of 39.9 %, 35.6 % and 28.7 % respectively. These values being below 50 % showed that they were distantly related since the

isolated virus could not bind strongly to their antisera.

The absence of secondary bands during the determination of molecular weight of the isolated *Tobamovirus* signified that the virus preparation was very pure containing no plant protein which would have shown another protein band on the gel. The value obtained for the molecular weight of the isolated *Tobamovirus* showed the virus to be a good immunogen that is able to trigger the formation of antibodies.

*In conclusion therefore, a strain of Tobamovirus purified from Mucuna pruriens through its susceptible host Vigna unguiculata var. TVu 76 showed that the molecular weight of the virus was 27kDa and the antiserum raised against this virus had a titre value of 1:2000. The virus was found to be closely related to TMV (Muguga strain) which was originally isolated from Kenya.*

## References

- Ayo-John, E. I., Olorunmaiye, P. M., Odedara, O. O., Dada, B. O., Abiola, K, O, and Oladokun, J. O, (2014). Assessment of field grown cucurbit crops and weeds within farms in southwest Nigeria for viral diseases. Not. Sci. Bio. 6: 321 - 325
- Clark, M. F. and Adams, A. N. 1977. Characteristics of microplate method of Enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Plt. Virol. 34: 574 - 586.
- Edwards, M.L. and Cooper J.I. (1985). Plant virus detection using a new form of indirect ELISA. J. Vir. Methd. 11: 309-319.
- Eni, A. O., Hughes, J.d;A. and Reys, M. E. C. (2008). Survey of the incidence and distribution of five viruses infecting yams in the major yam producing zones in Benin. Ann. Appl. Biol. 153: 223 - 232.
- Gibbs, A. J. and Harrison, B. D. (1976). Plant Virology- The Principles. Edward Arnold, 292 pp.
- Gooding (Jr.), J. V. and Herbert, T. T. (1967). A simple technique for purification of Tobacco mosaic virus in large quantities. Phytopath. 57: 1285.



- Hampton, R., Ball, E., De Boer, S. (1990). Serological Methods for Detection and Identification of Viral and Bacterial Plant Pathogens. A laboratory manual. Pp 33-54. APS Press. USA.
- Hughes, J.d'A. (1986). Viruses of Araceae and Dioscorea species. Their isolation, characterization and detection. Ph.D.Thesis. University of Reading, UK. 345 pp.
- Hughes, J.d'A., and Thomas, B. J. (1988). The use of protein-A sandwich ELISA as a means for quantifying serological relationships between members of the Tobamovirus group. *Ann. Appl. Bio.* 112: 117-126.
- Kareem, K. T., Odu, B. O., Umeh, V. C., Chikaleke, V.A., Oke, K. E., Arogundade, O, Adediji, A. O. and Odedara, O.O. (2014). Incidence and distribution of Citrus tristeza virus in citrus cultivars in Ibadan, southwest Nigeria. *J. Appl. Horti.* 15: 91-94.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nat.* 227: 680-685.
- Makau, M. and Gachene, C. K. K. (2001). Introduction of green manure legume cover crops to smallholders in Kimutwa location, Machakos District, Kenya. In: Legume Research Network Project Newsletter No. 5. [http://ppathw3.cals.cornell.edu/mba\\_project/CIIEPA/Impnews5.pdf](http://ppathw3.cals.cornell.edu/mba_project/CIIEPA/Impnews5.pdf)
- Matthews, R. E. F. 1991. *Plant Virology*. Third Edition. Academic Press. San Diego. California. 835 pp.
- Odedara, O. O., Hughes, J. d'A. and Ayo-John, E. I. (2007). Diagnosis, occurrence and seed transmission studies of viruses infecting *Centrosema* species in Nigeria. *Trop. Sci.* 47: 244 - 252.
- Odedara, O. O., Ayo-John, E. I., Gbuyiro, M. M., Falade, F. O. and Agbegbi, S. E. 2012. Serological detection of yam viruses in farmers' fields in Ogun State, Nigeria. *Arch. Phytopath. and Plt. Prot.* 45: 840 - 845.
- Odu, B.O., Hughes, J.d'A., Shoyinka, S.A. and Dongo, L.N. (1999). Isolation, characterization and identification of a potyvirus from *Dioscorea alata* L. (Water yam) in Nigeria. *Ann. of Appl. Bio.* 134: 65-71.
- Sanginga, N., Ibewiro, B., Hougandan, P., Vanlauwe, B., Okogun, J.A., Akobundu, I. O. and Versteeg, M. V. (1996). Evaluation of symbiotic properties and nitrogen contribution of *Mucuna* to maize growth in the derived savannah of West Africa. *Plt. Soil* 179: 119 - 129
- Scholthof, K. B. G. (2000). Tobacco mosaic virus. *The Plant Health Instructor*. DOI: 10: 1094/Plt I-1-2000-1010-01. Accessed May, 2016
- Versteeg, M. N. and Koudokpon, V. (1993). Participative farmer testing of four low external input technologies to address soil fertility decline in Mono province (Benin). *Agricult. Syst.* 42: 265 -276.

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