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DETERMINATION OF PROPERTIES OF A STRAIN OF TOBAMOVIRUS FROM *MUCUNA PRURIENS*

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ABSTRACT

A virus-infected leaf of *Mucuna pruriens* was collected and tested for an incidence of a virus through biological properties using various plant host range inoculation tests, longevity-in-vitro, dilution-end-point, thermal-inactivation point and electron microscopy. Results of host range inoculation tests showed the following plant species to be susceptible to the virus; *Nicotiana tabacum*, *N. rustica, N. benthamiana, N. glutinosa, N. occidentalis, N. Sylvesris* (Speg and Comes) and others.

No plant in the family Convolvulacaee, Cucurbitaceae was found to be infected with the virus. However *Chenopodium quinoa* and *C. murale* in the family Chenopodiaceae were infected with symptoms of mottling and necrotic lesions. In the family Fabaceae, hybrid cowpea line TVu 76, IT84S – 2114 were infected with mosaic, leaf crinkling and severe leaf curl respectively. The virus had a dilution-end-point of 10^{-6}-10^{-7}, thermal-inactivation-point of 95°C in crude sap of TVu 76. The virus is of agricultural importance because of its incidence on seed coats of legumes which are freely exchanged between agricultural stakeholders.

Keywords: Incidence, *Tobacco mosaic virus*, necrosis, mottling, inactivation

INTRODUCTION

The virus, *Tobacco mosaic Virus* (TMV) is a rigid rod-shaped virus which is non-enveloped and belonging to the Genus *Tobamovirus* (Alishiri *et al*., 2013). It had been found to infect many leguminous plants such as *Peuraria phaseoloides* and *Mucuna pruriens* (Hughes and Tarawali, 1989; Gumedzoe, 1996). Some other plants such as *Canavalia ensiformis*, *Cajanus cajan* and *Centrosema brasilianum* have also been found to be infected with the virus. It had also caused serious economic impact and significant yield losses in many crops such as Brassicas, Cucurbits, Solanaceous crops and different ornamental plants such as chrysanthemums (*Chrysanthemum indicum* L.), impatiens (*Impatiens balsamina*) and petunia (*Petunia hybrida*)(Alexandre *et al*., 2000; Choi *et al*., 2009; Kumar *et al*., 2011; Nassar *et al*., 2012). Some of the symptoms induced by TMV include severe stunting, leaf mosaic, mottling, distortion, chlorotic, mosaic and many others (Hughes and Tarawali, 1989; Marys *et al*., 2004). Tobacco mosaic virus had been known to cause disease on pepper and tomato (Scholthof, 2000; Pazarlar *et al*., 2013) The disease caused by TMV on pepper is one of

the major limiting factor affecting production of pepper in Turkey and all over the world, in both greenhouse and open field (Pazarlar *et al,.* 2013).

Mucuna is a vigorously growing, trailing vine. It is known as the velvet bean in Australia, United States of America and southern Africa; and as Bengal beans in India. Its importance have been studied by many and it is known to increase maize grain yield, improves soil physical, chemical and biological properties, prevent erosion and suppreses spear grass (Versteeg & Koudokpon, 1993, Sanginga *et al.*, 1996; Makau, 2001). Seeds of Mucuna have been shown to contain L-Dopa, a catecholic amino acid 3-(3,4-dihydroxyphenyl)-L-alanine, a neurotransmitter precursor that has found wide application for symptomatic relief of Parkinson disease and mental disorder (Faisal *et al.*, 2006). After the discovery that Mucuna seeds contain L-Dopa, its demand in the international market has increased many fold (Farooqi *et al.*, 1999). It grows in bushes in the southwestern Nigeria and bears pods that are itchy in nature. Its local name is 'werepe', because of the itchy nature. It is affected by a virus with symptoms of mosaic, leaf yellowing and curling. Due to its various importance to mankind, it is very imperative that a research be undertaken to characterize the virus infecting the plant and study its biological properties through host range susceptibility tests and electron microscopy. Knowledge about the properties of this virus is of great importance in acquisition of information that will be of assistance in the deployment of control strategies to be set up for this valuable plant.

MATERIALS AND METHODS

Host range tests

A virus-infected Mucuna leaf was collected from *Mucuna pruriens* growing among other herbaceous legumes planted in the seed multiplication plot of International Livestock Research Institute (ILRI) located inside the campus of International Institute of Tropical Agriculture, Ibadan. The virus-infected plant was macerated in a sterile mortar and pestle with inoculation buffer (0.1M phosphate buffer pH 7.7) containing 0.01M ethylene diaminetetracetic acid (EDTA) and 0.01M cysteine) at a ratio 1:10 (w/v). The extracted sap was inoculated onto the carborundum-dusted leaves of cowpea (*Vigna unguiculata var*. TVu 76) and other test plants so as to determine the host range. Excess inoculum on the leaves were washed off with tap water from a wash bottle to avoid leaf burns from left over carborundum. Inoculated plant species were labelled and kept in the screenhouse for about 4 weeks for symptom expression. Non inoculated plant species were labelled and kept as negative controls. The screehouse was sprayed weekly with Sherpa plus (280 EC) insecticide (Aventis Crop Science S. A France) at a concentration of 5ml/L to control insects.

Determination of Longevity-in-vitro of the virus isolate.

Longevity-in vitro (LIV) is defined as the time (expressed in weeks, days or hours) for which crude juice of an infected plant kept at room temperature remains infective. The test was carried out according to the method of Hill, (1984). LIV was determined for the Tobamovirus using the extracted sap of infected *Vigna unguiculata var*. TVu 76. The experiments were carried out in an insect-proof screen house sprayed weekly with Sherpa plus insecticide. Crude sap extract of two-week old infected leaves of TVu 76 was

prepared by homogenizing 10g infected leaves in a chilled mortar and pestle with 100 ml of 0.01M phosphate buffer pH 7.7. 2ml of the sap was pipetted into sufficient number of test tubes (PYREX, USA) each of 20ml in capacity corresponding to the chosen range of time intervals and the tube sealed with a parafilm. Tubes were left on racks in the laboratory at 28^{o}C away from direct sunlight and other variable heat or cooling influences. At the prescribed interval, each tube was opened and inoculated onto five healthy assay hosts in duplicate. Control plants of assay hosts inoculated with buffer only were set aside from the infected plants and were observed for a period of 2 to 4 weeks. The day or time interval at which symptoms were last observed was recorded.

Determination of Dilution-end-point

Dilution end point is the range of two dilutions i.e. the highest dilutions at which the sap of infected plant is still inefective and the next highest one where it becomes infective (Nordam, 1973). Crude sap of an infected plant was prepared as described earlier and a range of dilutions prepared by pipetting 1ml of extract with a Pipetmann (Gilson, France) of 1000 µl capacity, into the first of ten tubes (each of 20ml capacity (PYREX, USA). Into the remaining 9 tubes, each containing 9 ml of sterile water, 1 ml sap pipette from the first tube was transferred to tube 2, mixed well to give a dilution of 1/10. From tube 2 containing 1/10 dilution, 1 ml of the extract was transferred into tube 3 to give 1/100 dilution and this was repeated for the remaining tubes till the dilution 10^{-9} was obtained. Inoculation of each dilution was done to five healthy host plants of assay hosts in duplicate. Equal numbers of assay hosts inoculated with undiluted sap extract served as controls. Plants were observed for a period of 2 – 4 weeks for symptom expression and the dilutions between which infectivity was lost were recorded for each virus.

Determination of Thermal Inactivation point

According to Hill (1984), it is the temperature required for complete inactivation of virus following a ten minutes exposure of infected sap to heated water of required temperature in a bath. Crude sap extract of two weeks old infected leaves of TVu 76 were prepared by homogenizing 10g infected leaves in a diluted sterilized mortar and pestle with 100 ml 0.01M phosphate buffer (pH 7.7). Two ml of the infected sap extract was pipetted into each of 10 thin-walled test tubes each of 20 ml capacity was immersed into a water bath that had been heated to the required temperature (35^{o}C) for 10 min. Contamination of the tube walls was avoided during pipetting and the tubes were immersed in water so that the level of water was up to 3 cm above the level of sap in the tube. The first tube was removed, cooled in running water labelled with appropriate temperature and set aside for host assay. Successive tubes were treated as described, raising the temperature by 5^{o}C between each up to a maximum of 100^{o}C. Five plants (in duplicate) of *Vigna unguiculata* (cv. TVu 76) were inoculated with each treated sap. Inoculated plants were observed for a period of 2 – 4 weeks in the screenhouse. Further set of 5 plants inoculated with untreated sap were set aside as controls. The two temperature between which complete inactivation occurred were quoted for the virus.

Electron microscopy

Sap of infected leaf sample of TVu 76 containing the suspected virus was examined under a transmission electron microscope

(Model EM 208, Phillips Electron Optics, Netherlands) for presence of virus particles by the leaf dip method described by Odu *et al.,* (1999). A drop of 20 µl of potassium phosphotungstate KPT pH 6.8 was applied as a stain on a clean microscope slide and a piece of infected leaf tissue (about 1cm^2) placed on top of the drop. This was crushed in the stain with a glass rod. A drop of the extract with the stain (10 µl) was put onto a carbon-coated electron microscope copper grid (3.05 mm, 200 mesh) supplied by Agar Scientific, UK.

Excess stain was removed by touching gently the edge of the grid with a filter paper; grid was air dried for about 10-15 min. Stained grids were later observed at X22,000 to X100,000 and the video prints of the particles taken. Particles dimensions were estimated using the magnification scale between X50,000 to X100,000. A string was placed on the scale to get the length and used to measure the diameter of ten randomly selected particles.

RESULTS

The virus isolated from *M. pruriens* caused a systemic mosaic, leaf malformations and chlorosis on leaves of the plant. It was very infectious on almost all the plants tested. Necrotic lesions were produced by this virus on leaves of *N. occidentalis* which belong to the family Solanaceae (Figure 1). It also produced systemic mosaic with leaf malformations in *N. benthamiana*. In plants belonging to family Fabaceae it caused leaf deformation and severe mosaic (Table 1). In *Glycine max* 3 wks after inoculation, it induced red necrotic lesions. The virus induced symptomless infection in *Lycopersicon esculentum* and this was confirmed by back inoculation to *Nicotiana glutinosa* where necrotic lesions were observed 2 weeks later.

The virus was heat tolerant, able to survive the temperature range of 10^{o}C to 95^{o}C. At this temperature it infected 22.2% of inoculated TVu 76 host plants with mild mosaic symptoms. However at 100^{o}C, the virus ceased to be infectious (Table 2).

For Longevity-in-vitro, the sap extract of TVu 76 containing the virus was still infective at 32 days. It produced systemic mosaic and leaf malformations on *V. unguiculata* (Table 3). Result of electron microscopy of the crude sap of infected *M. pruriens* showed rigid rod-shaped particles which have their length as 300nm and width as 16nm (Fig. 2).

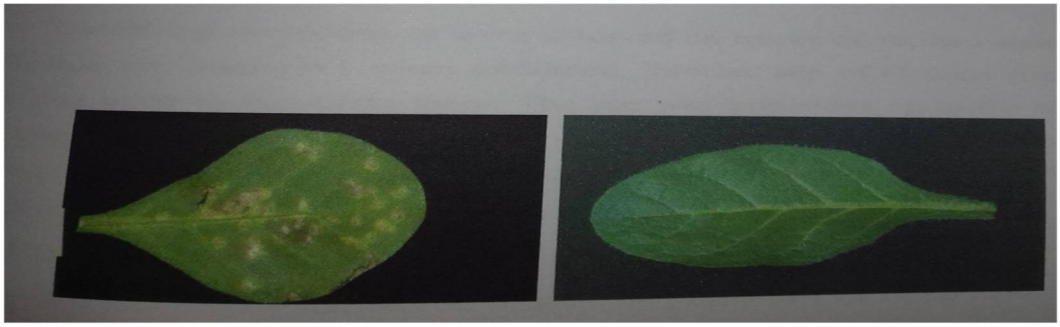

Figure 1: Symptoms of necrotic lesions caused by the strain of *Tobacco mosaic virus* on *Nicotiana occidentalis*

Table 2: Thermal inactivation point for the isolated Tobamovirus

Temperature (°C)	No of Plant inoculated	% Infection
35	10	90.0 ± 0.0
40	9*	100.0 ± 0.0
45	9*	100.0 ± 0.0
50	10	100.0 ± 0.0
55	10	100.0 ± 0.0
60	9*	100.0 ± 0.0
65	9*	100.0 ± 0.0
70	10	100.0 ± 0.0
75	10	100.0 ± 0.0
80	10	100.0 ± 0.0
85	10	100.0 ± 0.0
90	10	80.0 ± 0.0
95	9*	22.5 ± 0.0
100	10	0.0 ± 0.0

% = percentage, * some plants died prior to time of scoring.

Table 3: Longevity in vitro of the isolated Tobamovirus

Days after sap expression	Rate of infection	Percentage of virus transmission
5	10/10	100.0 ± 0.0
6	10/10	100.0 ± 0.0
8	10/10	100.0 ± 0.0
13	10/10	100.0 ± 0.0
15	10/10	100.0 ± 0.0
39	10/10	100.0 ± 0.0

Table 4: Dilution end point for the isolated Tobamovirus

Dilution	No of plant inoculated	Percentage infection
10^{-1}	10	100.0 ± 0.0
10^{-2}	10	100.0 ± 0.0
10^{-3}	10	100.0 ± 0.0
10^{-4}	8*	62.5 ± 8.8
10^{-5}	10	20.0 ± 0.0
10^{-6}	10	20.0 ± 0.0
10^{-7}	10	0.0 ± 0.0
10^{-8}	10	0.0 ± 0.0
10^{-9}	10	0.0 ± 0.0
Undiluted sap (Control)	10	100.0 ± 0.0

*some plant died prior to scoring.

Table 1: Symptoms induced by the isolated Tobamovirus on different host plants

Host plant	Symptom expression	Description of symptom
Solanaceae		
Nicotiana tabacum L.	+	n.l.
N. rustica L.	+	n.l.
N. benthamiana Domin	+	n.l.
N. occidentalis (Wheeler)	+	n.l.
N. sylvestris (Speg & Comes)	+	n.l.
Nicandra physaloides Gaertn	+	n.l.
Capscicum annuum L.	+	n.l.
C. frutecens L.	+	n.l.
Physalis floridana Rydb,	-	-
Datura stramonium L.	+	n.l. (sev)
Lycopersicon esculentum Mill	+	White striping
Convolvulaceae		
Ipomoea coccinea	-	-
Cucurbitaceae		
Cucumis sativus	-	-
Chenopodiaceae		
Chenopodium amaranticolor	+	c.l.+s.mott
Coste & Reyn		
C. quinoa Willd.	+	Mottr
C. murale L.	+	n.l.
Fabaceae		
Vigna unguiculata (L.) Walp		
cv Ife brown	+	mos +/v. curl
TVu 76	+	mos. +/v crk
TVu 2657	+	mos. + n.l
IT82E-10	+	mos. + n.l
Glycine max (L. Merr)	+	red n.l.
Malayan		
Aizoceae		
Tetragonia expansa (Murr)	+	n.l.

Key: + = symptom was observed; - = no symptom, n.l. = necrotic lesion

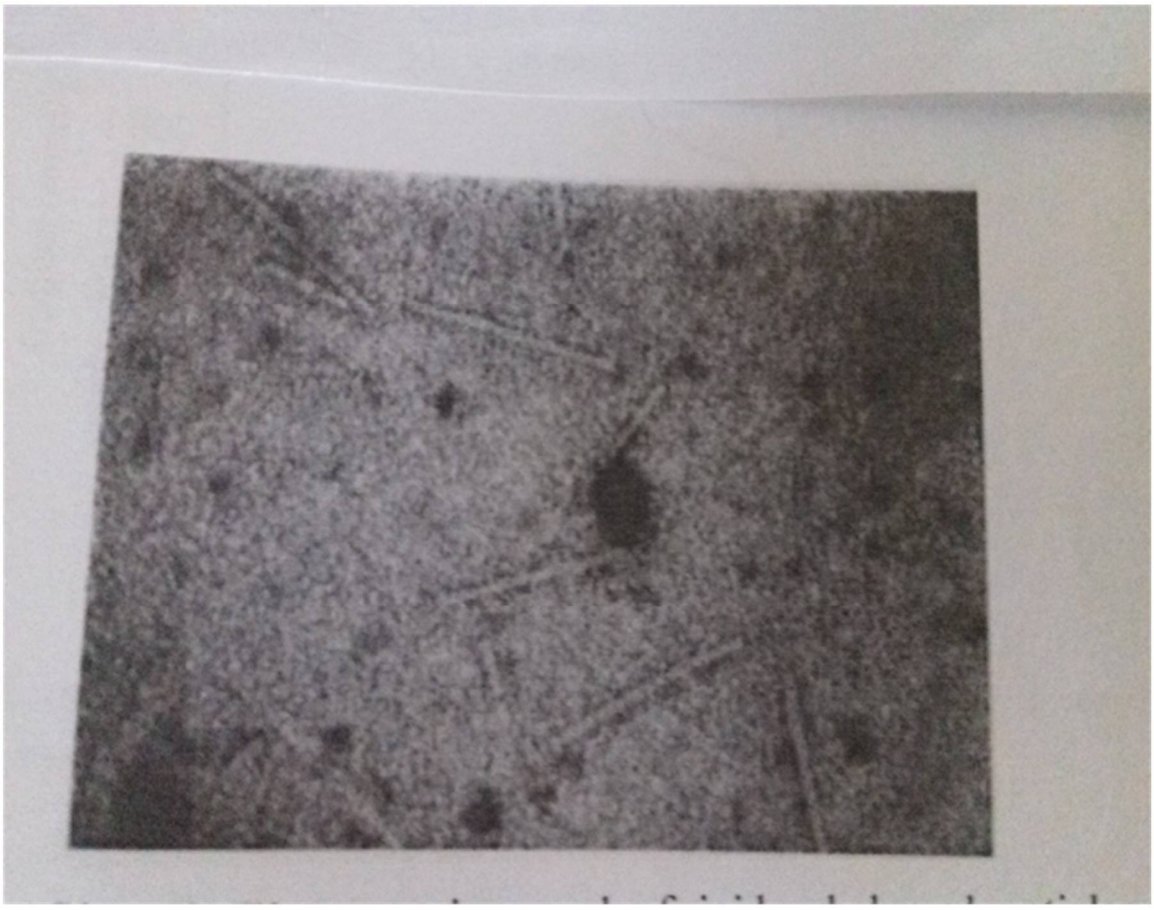

Figure 2 – Electron micrograph of the strain of Tobamovirus.

DISCUSSION

A virus isolated from *Mucuna pruriens* at IITA campus, Ibadan was kept on TVu 76 throughout the study. When reinoculated back to its healthy natural host, the virus isolate was found to be infective thus comfirm Koch's postulates. The virus was isolated from diseased vine of *Mucuna pruriens*. Similar virus had been isolated from ash plant in the United States of America (Lana & Agrios, 1974). There were similarities in the biological properties of the two viruses. For example, the infection of *N. glutinosa* with necrotic or local lesions by the virus were reported from both studies. However unlike the virus in this study which successfully infect soybean (*G. max*), strain isolated by Lana & Agrios (1974), failed to induce any visible symptom on *G. max*. Inoculation of a virus on different indicator hosts range is an important criterion in virus diagnosis. The high infectivity of the isolated virus might be explained as existence of different factors which influence the course of infection and disease during inoculations. Some of these factors are the concentration of the virus in the inoculum, genetic characteristics of the plant itself, such as plant age, host response and environmental factors such as light, temperature, relative humidity etc (Matthews, 1991). Absence of symptoms following inoculation of a particular test

plant may not probably mean that the virus was not infectious. Back inoculations to another indicator species might probably have revealed the presence of masked infection, which may not be uncommon (Matthews, 1991).

The physical properties of the virus such as Thermal Inactivation Point (TIP); Longevity-in-vitro (LIV) and Dilution End Point (DEP) showed that the virus is heat tolerant and can survive longer days in host plant and in a wider range of dilutions and still be infective. The DEP obtained from this study corroborates the findings of Lana and Agrios (1974) where the sap extracted from infected tobacco and *Chenopodium amaranticolor* had a DEP of 10^{-6} to 10^{-7} These values are still of great importance in routine handling of viruses, routine maintenance, infectivity tests and purification (Matthews, 1991).

Under electron microscopy, the virus isolated from *Mucuna pruriens* was found to be rigid rods belonging to family Tobamoviridae. This showed that the virus could be visualized using electron microscope. Filamentous rod-shaped viruses such as Potyviruses, Potexviruses and Tobamoviruses can be more readily differentiated in negatively stained leaf-dip preparations than isometric viruses and others (Naidu and Hughes, 2003). Electron microscopy is a basic requirement for virus identification using knowledge of the size, shape and surface features of the virus particles (Matthews, 1991). Apart from being a quicker method, it is also reliable.

In conclusion therefore, the virus isolated from *Mucuna pruriens* at IITA campus should be considered as a strain of Tobamovirus. These properties are still valuable in description of viruses infecting crops in Nigeria. In the temperate countries, researches have been done and biological and molecular data available for each of the existing viruses and plants infected. Further effort should be input for the full characterization of various viruses infecting all plants in Nigeria and a data bank established for future reference. Effort should also be made to eradicate the virus and others as their continue existence on natural host plant could pose a serious setback to the production of other crops such as *V. unguiculata* thereby reducing the yield as the latter plant is susceptible to the virus.

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