PROPERTIES OF A VIRUS ISOLATED FROM *VERNONIA AMYGDALINA* DEL. IN LAGOS, NIGERIA

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Received May 26, 2004; accepted November 3, 2004

Summary. – A previously uncharacterized virus tentatively named Vernonia green vein-banding virus (VGVBV) was isolated from *Vernonia amygdalina* Del. ("bitterleaf") from Lagos, Nigeria. The virus was mechanically transmissible but had a narrow host range restricted to *Nicotiana benthamiana, Chenopodium quinoa* and *C. amaranticolor.* It was also transmissible in a non-persistent manner by *Myzus persicae*. The virus was purified from *N. benthamiana* and about 750 nm long flexuous rod-shaped particles were observed in purified preparations as well as in leaf–dips of *Vernonia* sp. Inclusion bodies in the form of pinwheels and scrolls were observed in ultrathin sections of *Vernonia* leaves by electron microscopy. M_r of the viral coat protein was estimated to be about 34 K. In indirect ELISA, all 20 samples from naturally infected *Vernonia* sp. reacted positively with a potyvirus-specific monoclonal antibody (MAb) as well as with an antiserum raised against VGVBV. Apart from the homologous antigen, the VGVBV antiserum reacted only with Plum poxvirus (PPV). The VGVBV reacted strongly with the antisera to Bean yellow mosaic virus (BYMV), Bean common mosaic virus (BCMV) and Amaranthus leaf mottle virus (AmLMV) but weakly with antisera to PPV and Cowpea aphid-borne mosaic virus (CABMV) (all members of the family *Potyviridae,* the genus *Potyvirus*) in at least one of the assays used (indirect ELISA, dot-blot immunoassay and Western blot analysis). The results of our host range, cytopathological and serological studies and the available literature indicate that a hitherto difficult to transmit VGVBV has only been reported from Nigeria. We consider VGVBV a candidate for a new potyvirus. This virus should be further investigated to collect sufficient data for a qualified proposal of VGVBV as a new potyvirus.

Key words: *Vernonia amygdalina* Del.; Vernonia green vein-banding virus; potyvirus

Introduction

Vernonia amygdalina Del ('bitterleaf', the family *Asteraceae*) is a free branching perennial shrub widely cultivated as an edible vegetable in many parts of Africa (Hutchinson and Dalziel, 1963). In Nigeria, the leaves are of commercial value. They are used as condiments in soups, after the bitterness has been reduced by washing and steeping in water. The leaves and twigs are used in traditional medicine as antipyretic, hemostatic, antidiarrhoetic and antipruritic agent (Oliver, 1960; Owoyale *et al*., 1981). They also possess analgesic, antileishmanial and antimicrobial properties (Tella 1986; Tadesse *et al*., 1993). 'Bitter leaf' has also been reported to have a potential use as hop substitute in beer brewing (Aina and Ukpo, 1990). They are often used to demarcate compounds and farm units (Epenhuijsen, 1974).

There are very few reports of natural infection of *Vernonia* sp. by disease causing agents. Pruthi and Samuel (1942) have reported the infection of *V. cinerea* by Tobacco leaf

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Abbreviations: AmLMV = Amaranthus leaf mottle virus; BCMV = Bean common mosaic virus; BICMV = Blackeye cowpea mosaic virus); BGMV = Bambara groundnut mosaic virus; BYMV = Bean yellow mosaic virus; CABMV = Cowpea aphid-borne mosaic virus; EGMV = Eggplant green mosaic virus; ESMoV = Eggplant severe mottle virus; MAb = monoclonal antibody; PRSV = Papaya ringspot virus; PVMV = Pepper veinal mottle virus; PCB = phosphate-citrate buffer; p.i. = post infection; PPV = Plum poxvirus; PVMV = Pepper veinal mottle virus; TeMV = Telfairia mosaic virus; VGVBV = Vernonia green vein-banding virus; WMV-2 = Watermelon mosaic virus 2

curl virus in India. Albrechtsein *et al*. (1978) have also reported a mycoplasma-incited disease called *Vernonia* phyllody in *V. cinerea* in India.

In 1991, an uncharacterized mechanically transmissible virus with a wide host range has been reported on *Vernonia* sp. from Southern Nigeria (Atiri and Osemobor 1991).In 1993, about 72% of the *Vernonia* plants cultivated in Lagos, Nigeria were reported to be infected with a virus that was difficult to transmit mechanically and that had a host range restricted to *V. amygdalina*. The virus was tentatively named VGVBV on the basis of symptomatology and the presence of flexuous rod-shaped particles of about 750 nm in length in leaf-dips of *Vernonia* sp. (Shoyinka *et al*., 1993). VGVBV has not been so far recognized as a virus by the International Committee on Taxonomy of Viruses (ICTV) (van Regenmortel *et al.,* 2000*).*

Due to the very scanty information on viral diseases of *Vernonia* sp. and because of the economic importance of "bitterleaf", this investigation was conducted to characterize it and to determine its serological relationship to other members of the family *Potyviridae*. An abstract has been published earlier (Taiwo and Dijkstra, 1996).

Materials and Methods

Virus isolation, host range and vector transmission studies. VGVBV was isolated from naturally infected *Vernonia* leaves. Young symptomatic leaves were ground in 0.01 mol/l phosphate buffer pH 7.2 containing 0.01 mol/l sodium diethyldithiocarbamate. The virus obtained was mechanically transmitted to *Chenopodium quinoa* Willd and *C. amaranticolor* Coste and Reyn. The virus was subsequently maintained on *Nicotiana benthamiana* Domin after three successive single lesion transfers on *C. quinoa*. The host range was determined by inoculating at least five plants of each of 17 plant species with VGVBV after dusting their leaves with carborundum. The plants tested included *V. amygdalina* Del.*, N. benthamiana, C. quinoa, C. amaranticolor, Amaranthus hybridus* L.*, Capsicum annuum* L. (sweet pepper), *Phaseolus vulgaris* L. (Bataaf), *P. lunatus* L.*, Pisum sativum* L.*, Citrullus vulgaris* L.*, Cucurbita pepo* L.*, N. glutinosa* L.*, N. tabacum* L.*, N. clevelandii* Gray*, N. rustica* L.*, Vigna radiata* L. Wilczek and *V. unguiculata* L. Walp. The inoculated plants were kept in greenhouse at about 25°C for four weeks for symptom development. Symptomless plants were checked by a dot-blot immunoassay for latent infection. The vector transmission study was performed with non-viruliferous adults of *Myzus persicae* Sulz. The aphids were starved for 2 hrs and allowed to feed on detached leaves of *N. benthamiana* for 5 mins. The aphids were transferred singly or in groups of fives to 10 healthy *N. benthamiana* plants for inoculation feeding for 1 hr. The plants were sprayed with 0.5 g/l Pirimor and kept in greenhouse at 20–25°C for symptom development. Electron microscopy of leaf-dip was used to confirm virus presence in symptomatic plants.

Virus purification, cytopathology and coat protein studies. The virus was purified from leaves of *N. benthamiana* on days 17–21 post infection (p.i.) according to Taiwo and Gonsalves (1982). The yield of purified virus was estimated with Gilford 2400-2 spectrophotometer using an extinction coefficient of 2.4 at 260 nm according to Purcifull (1966). Virus particles from the purified virus preparation and leaf-dips from infected *Vernonia* leaves were negatively stained with 2% phosphotungstic acid pH 6.0 and examined under a Zeiss E.M. 109 electron microscope.

Ultrathin sections of infected *Vernonia* leaves were prepared for cytopathology by cutting the leaves into pieces, fixing in 3% glutaraldehyde and 2% paraformaldehyde in 0.1 mol/l phosphate-citrate buffer pH 7.2 (PCB) for 2–3 days. The tissues were further washed with PCB, fixed in 1% osmium tetroxide for 1hr at 25°C, washed again with PCB and then dehydrated through 30%–100% ethanol. The samples were infiltrated with propylene oxide and propylene oxide plus epon 812 for 48 hrs and were sectioned with an ultramicrotome using glass knives. The sections were stained with 5% uranyl acetate and examined under Phillip CM 12 electron microscope.

M_r of the coat protein of VGVBV was estimated by SDS-PAGE according to Laemmli (1970). Protein size markers with M_r between 14.4 K and 94 K were included.

Serology. A polyclonal antiserum against VGVBV was raised by injecting 200 µg of purified virus emulsified with 1.0 ml of Freund's incomplete adjuvant subcutaneously (s.c.) into a New Zealand white rabbit. After 2 weeks 800 µg of emulsified purified virus was again injected into the rabbit. The latter was bled 18 days after and the blood was processed for antiserum according to Taiwo and Gonsalves (1982). The antiserum produced and a potyvirus-specific MAb (Agdia Inc., USA) were used to confirm the identity of the purified virus as well as to ascertain the virus status of 20 symptomatic samples of *Vernonia* sp. collected in Lagos, by an indirect ELISA.

In order to determine the serological relationship between-VGVBV and other potyviruses, reciprocal serological tests were conducted when possible, using indirect ELISA, dot-blot immunoassay and Western blot analysis according to van Regenmortel and Burckard (1980); Bantarri and Goodwin (1985) and O'Donnell *et al.* (1982). The antisera used in the tests were obtained from the Agricultural University, Wageningen, Netherlands. They included antisera raised against the following potyviruses: BYMV, BCMV, Blackeye cowpea mosaic virus (BICMV), PPV, AmLMV, CABMV, Papaya ringspot virus (PRSV), Watermelon mosaic virus 2 (WMV-2), PVMV, and Bambara groundnut mosaic virus (BGMV).

Results

Symptomatology, host range and vector transmission

The virus was difficult to transmit from infected to healthy *Vernonia* plants by mechanical inoculation. Transmission was easier with the purified virus. The VGVBV induced green vein-banding and mosaic in inoculated *Vernonia* plants. The symptoms were similar to those induced in the naturally infected plants (Fig. 1a).

In general, VGVBV had a restricted host range. The virus from *Vernonia* sap was readily transmitted to *N. benthamiana*

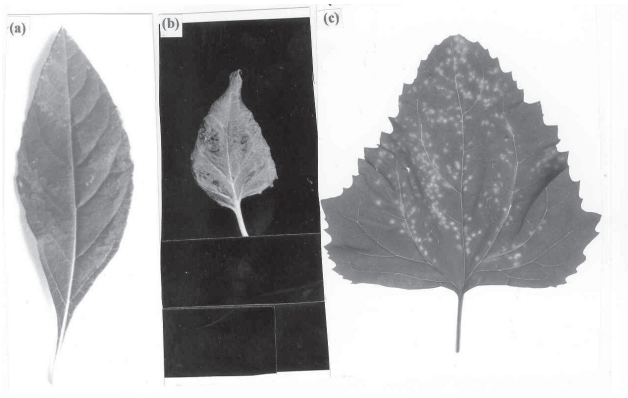

Fig. 1

Symptoms induced by VGVBV

Green vein-banding and mosaic in *Vernonia amygdalina* (a), mosaic and leaf curl in *Nicotiana bentamiana* (b), and chlorotic local lesions on *Chenopodium amaranticolor* (c).

on which it induced leaf curl and mosaic (Fig. 1b). It also induced local chlorotic lesions on *C. amaranticolor* and *C. quinoa* (Fig. 1c).

Necrotic lesions were also observed on the inoculated leaves of *Amaranthus* sp. None of the other plant species tested developed symptoms of infection and virus was not recovered from inoculated and uninoculated leaves by the dot-blot immunoassay (Table 1).

Table 1. Reaction of test plants to mechanical inoculation of VGVBV

Test plant	Symptoms	Result of the recovery test
Vernonia amygdalina *Del.*	M, GVB	+
Nicotiana benthamiana Domin.	Mo, LC	+
Chenopodium quinoa Willd	CLL-1	+
C. amaranticolor Coste and Reyn.	CLL-1	+
Amaranthus hybridus L.	NLL-1	–
Capsicum annuum L. (Sweet pepper)	NS	–
Phaseolus vulgaris L. (Bataaf)	NS	–
P. lunatus L.	NS	–
Pisum sativum L.	NS	–
Citrullus vulgaris L.	NS	–
Cucurbita pepo L.	NS	–
Nicotiana glutinosa L.	NS	–
N. tabacum L.	NS	–
N. clevelandii Gray	NS	–
N. rustica L.	NS	–
Vigna radiata L.Wilczek	NS	–
V. unguiculata L.Walp	NS	–

M = mosaic, GVB = green vein-banding, Mo = mottle, LC = leaf curl, CLL-1 = chlorotic local lesion on inoculated leaves, NLL = necrotic local lesions on inoculated leaves, NS = no symptom, (+) = virus recovered, (–) = virus not recovered by the dot-blot immunoassay.

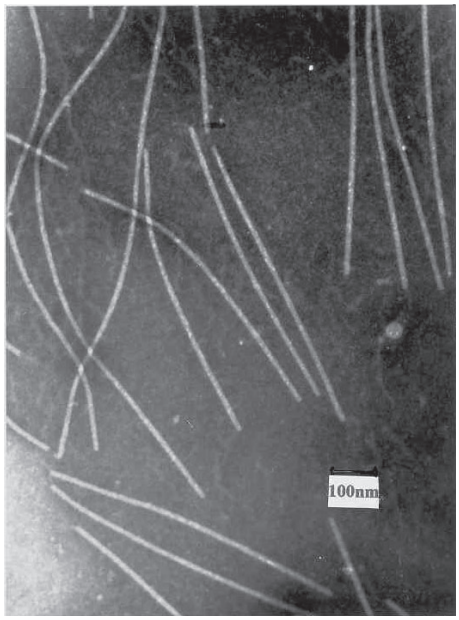

Fig. 2

Electron microscopy of a purified preparation of VGVBV showing

Flexous rod-shaped particles.

The virus was transmitted in a stylet-borne manner to two of the five plants that had five viruliferous aphids/plant. Flexuous rod-shaped particles of about 750 nm in length were observed in the leaf-dips of the two symptomatic plants.

Virus purification, cytopathology and coat protein studies

VGVBV was readily purified from the leaves of *N. benthamiana*.The yield of purified virus was about 4 mg/100 g of tissue. Flexuous rod-shaped particles of about 750 nm in length were observed in leaf-dips of infected *Vernonia* leaves and purified virus preparations (Fig. 2).

Inclusion bodies in the form of pinwheels and scrolls were observed in ultrathin sections of infected *Vernonia* leaves (Fig.3).

The viral coat protein was estimated to have a molecular mass of about 34 K (Fig. 4).

Serological studies

In serological studies all 20 symptomatic samples from naturally infected *Vernonia* sp. reacted positively with the potyvirus-specific MAb (Agdia Inc., USA) and an antiserum to VGVBV in ELISA.

In reciprocal serological tests with antisera to other potyviruses, VGVBV reacted strongly with the antisera raised

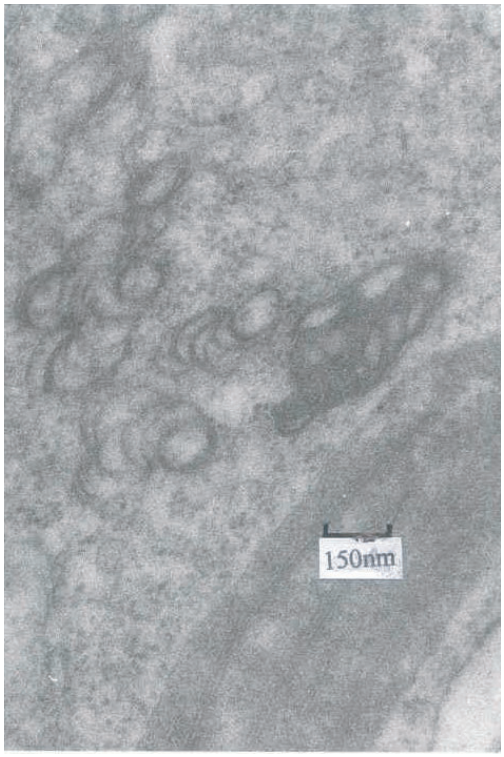

Fig. 3

Ultrathin section of infected *Vernonia* leaf

Cytoplasmic inclusions in the form of pinwheels and scrolls.

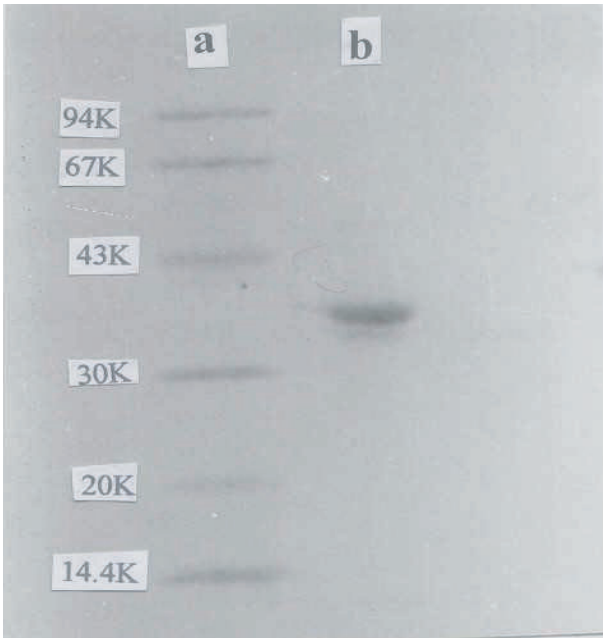

Fig. 4

SDS-PAGE of VGVBV coat protein

Marker proteins in the range of 14.4–94 K (lane a), VGVBV coat protein of 34 K (lane b).

to BYMV, BCMV and AmLMV but weakly with the antiserum to PPV in ELISA. It also reacted with the antisera to AmLMV, BCMV and CABMV in the dot-blot immunoassay and with the antiserum to AmLMV in Western blot analysis (Table 2).

The antiserum to VGVBV reacted weakly only with PPV in ELISA. Reciprocal serological reactions were only observed with PPV but they were very weak. The antiserum to AmLMV reacted strongly with VGVBV in all assays but the reactions were non-reciprocal.

Table 2. Reactions of VGVBV with the antisera to some potyviruses

Antiserum	Serological assay	
	ELISA	Dot-blot
BYMV	+++	–
BCMV-NY15	+++	++
BICMV	++	++
PPV	+	–
AmLMV	+++	+++
CABMV	–	+
PRSV	nt	–
WMV-2	nt	–
PVMV	nt	–
BGMV	nt	–
VGVBV	+++	+++

For the virus abbreviations see the list on the front page.
(+++) = very strongly positive, (++) = strongly positive, (+) = weakly positive, (–) = negative, nt = not tested.

Discussion

The positive reaction of VGVBV with the potyvirus-specific MAb, flexuous rod-shaped particles of the virus and the induction of pinwheels and scrolls as cytoplasmic inclusions indicated that the virus belongs to the *Potyviridae* family. The transmissibility of the virus mechanically and by aphids confirmed that it belongs to the *Potyvirus* genus (Shukla *et al.*, 1994).

Members and tentative members (yet to be recognized) of the *Potyviridae* family that have been reported to infect vegetables in Nigeria include Telfairia mosaic virus (TeMV, Nwauzo and Brown, 1975; Shoyinka *et al*., 1987), Eggplant green mosaic (EGMV) and Eggplant severe mottle virus (ESMoV, Ladipo 1976; Ladipo *et al*., 1988a, 1988b), Pepper veinal mottle virus (Lana *et al*., 1975; Ladipo and Roberts, 1977; Igwegbe and Waterworth, 1982), Amaranthus mosaic virus (Taiwo *et al*., 1987), Celosia mosaic virus (Atiri and Osemobor, 1991; Owolabi *et al*., 1998) and a so far uncharacterized virus on *V. amygdalina* that was tentatively named VGVBV (Atiri and Osemobor, 1991; Shoyinka *et al*., 1993). VGVBV was easy to transmit and it infected several

hosts (Atiri and Osemobor, 1991), while the isolate reported by Shoyinka *et al*. (1993) was very difficult to transmit. It was only transmissible to *Vernonia* sp. in the presence of additives such as sodium diethylthiocarbamate and cysteine hydrochloride. Its highly restricted host range made further investigation very difficult.

The isolate used in this study is believed to be similar to that reported by Shoyinka *et al*. (1993). VGVBV differs from the other potyviruses reported from Nigeria in host range and serological affinity. The widening of the host range of VGVBV observed may be due to single lesion culturing which might have led to the selection of such a variant. A similar phenomenon was observed for AmLMV by Lovisolo and Lisa (1976).

The results of the serological tests indicate that VGVBV may be related to PPV and AmLMV as well as to several other potyviruses. The cross- reactivity of the antisera raised to other potyviruses with VGVBV is not unusual. The variation in the specificity of the antisera may be due to differences in the extent of degradation of the purified viruses used for immunization. In addition, the immunization protocol employed as well as the differences in the interval between immunization and bleeding of the animals might have contributed to the lack of specificity (Koenig and Bercks, 1968; van Regenmortel and Von Wechmar, 1970). The antibodies may also have been directed against the conserved core region of the coat protein rather than against the virus-specific N-terminus of the coat protein. Such core region antisera are known to be capable of cross-reacting with many potyviruses (Richter *et al*., 1994). The highly limited reaction of the polyclonal antiserum raised against VGVBV might be due to the fact that the serum was produced from an early bleeding (about 4 weeks after the first injection) of the rabbit. Such early bleedings have been reported to be very specific and lacking cross-reactivity (Taiwo and Gonsalves, 1982; Owolabi *et al*., 1998).

The unilateral reaction of the antiserum to AmLMV with VGVBV in all the immunoassays suggests a close serological relationship, but AmLMV differs from VGVBV in host range and symptomatology. It induces systemic mosaic in *Amaranthus* sp., *C. amaranticolor* and *C. quinoa* while VGVBV induces local necrotic or chlorotic lesions in these hosts, respectively. However, both viruses are similar in their serological relatedness to BYMV and PPV (Lovisolo and Lisa, 1976, 1979).

Although VGVBV showed a weak reciprocal serological reaction with PPV, the latter is different from the virus under investigation in its natural host range, which is confined to *Prunus* sp. and in symptomatology and geographical distribution (Shukla *et al*., 1994).

The results of the study described here suggest that VGVBV may be a newly recognized member of the family *Potyviridae*. This assumpton is based on the particle morphology, biological, chemical, and serological properties as well as the restricted geographical occurrence of the virus.

We think that the virus should retain its present name (VGVBV) until crucial data like gene organization and genome and protein sequences will be available and an appropriate complex proposal of VGVBV as a new potyvirus (species) to the ICTV will be made.

The identity and taxonomic status of VGVBV will then be confirmed.

Acknowledgement. M.A.T. is grateful to the University of Lagos and the World Bank for the award of a fellowship that enabled her to spend 3 months in the Virology Laboratory of the University of Agriculture, Wageningen, The Netherlands and to Mr D. Lohuis for technical assistance.

Note of the Editor-in-Chief. To date, the International Committee on Taxonomy of Viruses does not list Amaranthus mosaic virus**,** Celosia mosaic virus, Vernonia green vein-banding virus (VGVBV) and among viruses (virus species) (van Regenmortel *et al*., 2000).

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