Identification of Potyviruses infecting forage grasses in Ethiopia

A.M. MIH[1] and J. HANSON[2]

[1]Department of Life Sciences, University of Buea, P.O. Box 63, Buea, CAMEROON.
[2]Conservation of Biodiversity Programme, International Livestock Research Institute, Addis Ababa, ETHIOPIA

ABSTRACT

During routine monitoring of field genebanks of forage grasses in Ethiopia to ensure the distribution of material of acceptable phytosanitary status, plants of *Pennisetum clandestinum, Pennisetum purpureum* and *Pennisetum purpureum x P. typhoides* showing virus-like symptoms were observed. Electronmicroscopic examination of leaf dip preparations revealed flexuous filamentous virus-like particles. The experimental host range closely resembled that of *Elephant grass mosaic virus* (EGMV) from Brazil. Purification of the virus isolates from *P. purpureum* (Is 14982) and *P. purpureum xP. typhoides* (Is 16840) yielded 76 mg and 84 mg virus per 1000g fresh leaf tissue, respectively. Antibodies produced in rabbits against these isolates had titres of 1/1024 and 1/512 for Is 14982 and Is 16840, respectively, in tube precipitin tests. Although the three virus isolates tested positive for JGMV (*Johnson grass mosaic virus*) in ELISA, samples from plants of *Digitaria milangiana, Paspalum auriculatum*, and seven accessions of *Paspalum scrobiculatum* naturally infected with JGMV tested negative for Is 14982 and Is 16840 in ELISA. Attempts to transmit the virus isolates using aphids were unsuccessful. Other viruses detected during the survey included MDMV (*maize dwarf mosaic virus*), SCMV (*sugarcane mosaic virus*) and JGMV. Of these viruses, JGMV is being reported for the first time from Ethopia.

Key words: Potyviruses, forage grasses, *Pennisetum*

RÉSUMÉ

Au cours d'inspections routinières de stocks d'herbes fourragères en champs pour s'assurer de la distribution de materiél de statut phytosanitaire acceptable, des plantes de *Pennisetum clandestinum, Pennisetum purpureum* et *Pennisetum purpureum x P. typhoides* présentant des symptômes d'infection apparemment virale ont été observés en Ethiopie. L'examen au microscope électronique de préprations de feuilles plongées a revélé la présence des particules filamenteuses en flèche, ayant l'aspect de virus. La série d'hôtes expérimentaux ressemblait étroitement à celle du virus de la mosaïque du Roseau (EGMV) du Brésil. L'isolation des souches virales de *P. purpureum* (Is 14982) et de *P.purpureum x P. typhoides* (Is 16840) a produit 76 mg et 84 mg de virus par 1000g de tissue de feuilles fraîches, respectivement. Les anticorps produits dans les lapins contre ces souches avaient des titres de 1/1024 pour Is14982 et 1/512 pour Is16840 dans des tests de précipitation en tube. Bien que les trois souches virales aient testé positif pour le virus de la mosaïque de l'herbe de Johnson en ELISA, les échantillons de *Digitaria milangiana, Paspalum auriculatum*, et sept accessions de *Paspalum scrobiculatum* naturellement infecté par le JGMV ont présenté des réactions négatives pour Is14982 et Is16840 en ELISA. Des essais de transmission de ces souches virales par des pucerons ont été vains. D'autres virus détectés au cours de ces inspections incluent: le virus de la mosaïque naine du maïs (MDMV) , le virus de la mosaïque de la canne à sucre (SCMV) et le JGMV. De ceux-ci, le JGMV est ennoncé pour la première fois en Ethiopie.

Mots clés: potyvirus, herbes fourragères, *pennisetum*

INTRODUCTION

Grasses are very important in the restoration of soil productivity during fallow and as a grazing resource. The cultivation of elephant grass, *Pennisetum purpureum* Schumach, for fodder has become popular in East Africa in the sustenance of the small-scale dairy farms (Boonman, 1993). The selection and improvement of grasses has thus become imperative. He International Livestock Research Institute (ILRI) maintains a large collection of grasses in trust in its genebanks with the aim of making propagation material freely available for evaluation and subsequent incorporation into sustainable agro-pastoral systems. Since many grasses hardly set viable seeds, vegetative materials are often distributed. There is thus the risk of spreading viruses and other diseases to distant areas where these did not exist (Hampton et al. 1982). The field genebanks of the ILRI are therefore routinely monitored for diseases to ensure that only propagative material of acceptable phytosanitary standards is distributed. Three unfamiliar virus isolates were found during routine surveys. Results of partial characterization of these virus isolates and the prevalence of other potyviruses are reported in this paper.

MATERIALS AND METHODS

Virus Isolates

Three virus isolates were studied. These are Is 16840 from plants of *P. purpureum* x *P. typhoides* hybrid accession 16840 showing elongated chlorotic lesions, Is 14982 from *P. purpureum* accession 14982 showing mild mottle symptoms, and Is Kikiyu from *P. clandestinum* plants growing on field paths and exhibiting symptoms similar to Is 16840. All virus isolates were from fields located at Debre Zeit, Ethopia with an altitude of 1850m. The virus isolates were maintained in plants raised from vegetative material of their respective original field hosts.

Host range studies

Forty-eight accessions of 15 grass species were mechanically inoculated with infective sap of each of the three virus isolates. The inoculum was prepared by triturating infected tissue of virus maintenance host in cold 0.01 M phosphate buffer, pH 7.2 (1g tissue per 5mL buffer). Plants for inoculation were produced from seeds except for *Cynodon dactylon, P. purpureum* and *P. purpureum* x *P. typhoides* where plants derived from *in vitro* culture of meristem tips were used. This was due to the unavailability of viable seeds of these species. Inoculated plants were placed in an aphid-proof screenhouse (temperature 19-30 ^{0}C) and observed for symptoms for up to 30 days.

Serology

The viral isolates were tested serologically by the antigen coated plate indirect enzyme-linked immunosorbent assay (ACP-ELISA) by standard methods (Hill, 1985) against polyclonal antisera to the following viruses: *Barley stripe mosaic virus* (BStMV) from the Danish Government Institute of Seed Pathology for Developing Countries; *maize streak* (MSV), *maize mosaic* (MMV) and *maize stripe viruses* (MStV) from CITRAD, France; *barley yellow dwarf mosaic virus* (BYDMV) from Agdia Inc. USA, *rice yellow mottle virus* (RYMV) from IITA Nigeria; *elephant grass mosaic virus* (EGMV) from Prof. Kitajima, Brazil; *Maize dwarf mosaic virus* (MDMV), *sorghum mosaic virus* (SrMV), *sugar cane mosaic virus* (SCMV) and *Johnson grass mosaic virus* (JGMV) from IAR, Ambo, Ethopia. Samples of the test isolates were extracted in 0.05M carbonate buffer, pH 9.6, containing 2% (w/v) polyyinlpyrrolidone and 0.1% Na_2SO_3 (w/v) at the rate 1 g/10mL buffer. Antibodies were diluted as per the specifications of the suppliers. All antibodies were adsorbed for 2 to 3 h at room temperature with healthy maize sap in phosphate buffered saline, PBST, (137mM NaCl, 1.5mM KH_2PO_4, 3mM KCl, 3mM NaN_3 0.05% v/v Tween 20) at the rate of 1g tissue per 100mL buffer. Goat antirabbit alkaline phosphatase conjugate (Sigma co.) was diluted 1/2000 in PBST. The ρ-nitrophenylphospate substrate was used at the rate of 1 mg/mL 10% diethanolamine, pH 9.8 (substrate buffer). Plates were read with a BioRad EIA reader (model 2550) with the substrate buffer as a blank.

Virus purification ad antibody production

Is 16840 and 14982 were purified from vegetatively multiplied material of the respective maintenance host. Leaf tissues were extracted in cold 0.5 M phosphate buffer pH 7.5 containing 0.75% Na_2SO_3 (w/v), 0.01M EDTA and 3% v/v TritonX- 100. The homogenate was strained through four layers of cheese cloth after which n-butanol/choloroform (1:1) was added to a final concentration of 30% (v/v). The mixture was stirred for 15 min at room temperature, then centrifuged at 7000g for 15 min. Polyethyleneglycol (MW 8000) was added to the supernatant (8% w/v) and the mixture was stirred for 45 minutes at 4 ^{0}C to precipitate the virus. The precipitate was recovered by centrifuging at 7500g for 15 min, and resuspended overnight at 4 ^{0}C in 0.01 M borate buffer pH 8.3

containing 0.001 M EDTA (suspension buffer). The resuspended virus was dispersed by pocking in a Griffit's tube. The suspension was centrifuged at 7500g for 15 min. The supernatant was layered on a 20% (w/v) sucrose cushion in the suspension buffer and centrifuged at 21000g for 180 min. the pellet was suspended in five times the original volume in the suspension buffer and passed through three cycles of differential centrifugation at 21000g for 180 min. The virus yield was estimated using an extinction coefficient of 2.35 (Brunt et al., 1990).

Rabbits were given two weekly intravenous injections (unemulsified), followed by three weekly intramuscular injections of 1mL virus (concentration cf. 1 mg/mL emulsified with an equal volume of Freud's incomplete adjuvant). The rabbits were bled 10 days after the last injection and the antibody titre determined by standard methods (Hill, 1984). Weekly bleeding was done and booster injections of emulsified virus were given when the antibody titres were observed to be falling. Partial purification of IgG was done by the method of Clark and Adams (1977) except that the filtration step through Whatman DE 22 column was omitted.

Survey for other grass viruses

A sero-diagnostic survey of newly established plots of some grasses in the field genebank at the Debre Zeit Research Station was done using our prepared antisera along with antisera used for the serological assay of the three *Pennisetum* virus isolates. Only samples from plants showing virus-like symptoms were used for the serological assay by ACP-ELISA

RESULTS

The virus isolates Is 14982 and Is Kikuyu were transmissible only to *Pennisetum* sp. and *Zea mays* (Table 1). Is 14982 incited mild mosaic on both *P. purpureum* and the maize cultivar Findish. The symptoms disappeared with aging of the plants. Symptoms due to Is 16840 were a persistent severe mosaic resulting in stunted plants. Is Kikuyu caused chlorotic mottle in maize cv Findish, but infected *P. purpureum* symptomlessly. Only Is 16840 infected *P. polystachyum*, causing yellow mottle and leaf curl.

All three isolates tested positive for *elephant grass mosaic* and *Johnson grass mosaic viruses* (Table 2). The absorbance values for Is 14982 in the positive tests were generally lower than those for either Is 16840 or Is Kikuyu.

The purification protocols yielded 76 mg and 84mg virus per 1000g leaf tissue for Is 14982 and Is 16840, respectively. The antibody titres in tube precipitin tests were 1/1024 for Is 14982 and 1/512 for Is 16840. Both antibodies detected all three virus isolates to different extents.

The reaction of field samples to antibodies of some grass viruses is shown in Table 3. Of the 35 samples tested none reacted positively to any of the antibodies

Table 1. Partial experimental host range of three *Pennisetum* virus isolates.

Test Plant	No. of Accessions Inoculated	No. Accessions Infected by virus		
		Is 14982	Is 16840	Is kikuyu
Avena sativa	1	0	0	0
Brachiaria brizantha	2	0	0	0
Brachiaria decumbens	1	0	0	-
Brachiaria humidicola	1	0	0	-
Brachiaria roziziensis	1	0	0	-
Cynodon dactylon	1	0	0	0
Eragostis teff	1	0	0	0
Hordeum vulgare	3	0	0	0
Panicum maximum	2	0	0	0
Pennisetum polystachyum	6	0	1	0
Pennisetum purpureum	1	1	1	1
P. purpureum x.P. typoides	1	1	1	1
Sorghum bicolor	15	0	1	0
Triticum aestivum	2	0	0	0
Zea mays	10	1	2	1

- = Not tested

Table 2. Serological detection of three *Pennisetum* virus isolates by ACP-ELISA

Virus Antibody[x]	Reaction of Antigen[y]			
	Is 14982	Is 16840	Is kikuyu	Healthy Sap
BStMV	0.056	0.059	0.027	0.043
BYDMV	0.031	0.028	0.052	0.029
EGMV[z]	0.408	1.964	0.938	0.036
JGMV[z]	1.386	2.000	2.000	0.024
MDMV	0.083	0.075	0.061	0.052
MMV	0.031	0.008	0.021	0.009
MSV	0.043	0.051	0.048	0.043
MStV	0.051	0.050	0.062	0.048
RYMV	0.008	0.011	0.180	0.010
SCMV	0.061	0.017	0.036	0.008
SrMV	0.013	0.026	0.080	0.031

[x] Antibodies to *barley stripe mosaic* (BStMV), *barley yellow dwarf* (BYDMV), *elephant grass mosaic* (EGMV), *Johnson grass mosaic* (JGMV), *maize dwarf mosaic* (MDMV), *maize mosaic* (MMV) *maize streak* (MSV), *Maize stripe* (MStV), *rice yellow mottle* (RYMV), *sugarcane mosaic*(SCMV) and *sorghum mosaic* (SrMV) viruses.

[y] Is 14982 = virus isolate from *P. purpureum*, Is 16840 = Isolate from *P. purpureum x P. typhoides*, Is Kikuyu = isolate from *P. clandestinum*, Healthy sap = healthy sap from maize. Values are absorbance at 405nm read 30 min after application of substrate (mean of 10 replicates).

[z] Virus isolates tested positive with these antibodies.

BStMV, MSV, MStV, MMV, BYDMV, RYMV and SrMV in ACCP-ELISA. Most of the samples tested positive for *Johnson grass mosaic virus*, while only one sample from *Urochloa panicoides* tested positive for MDMV. All accessions of *Pennisetum ramosum* tested positive for the *Pennisetum* virus isolates and for *Johnson grass mosaic virus.*

DISCUSSION

The reaction of test plants to the virus isolates from *Pennisetum* species and hybrid closely resembled that of EGMV reported from Brazil (Martin & Kitajima, 1993), suggesting a close relationship. This was confirmed by the positive reaction of the isolates to antibodies against EGMV in ELISA. However, the Brazilian isolate could not be transmitted back to elephant grass while those in this study were. This may be due to the fact that *in vitro* plantlets used for inoculation in this study were tender and this increased susceptibility. The *Pennisetum* virus isolates reacted strongly with antibodies to *Johnson grass mosaic virus* in serological assay while the Brazilian isolates of EGMV did not. The ELISA was used in this study while Martin and Kitajima (1993) used gel diffusion test in their serological assay. Preliminary tests by gel diffusion in our lab were not positive for our isolates either. The difficulty in diffusion of potyviruses even after disruption with SDS may account for this (Hill, 1984). Although our virus isolates reacted with antibodies to JGMV, the host range differed from that reported for JGMV (Brunt et al., 1990). This was also evident in the field, where accessions of *Paspalum scrobiculatum* and *P. auriculatum* testing positive for JGMV were negative for either of Is 14982 and Is 16840.

The three virus isolates were not identical as seen from serological reactions and host range studies. Is Kikuyu is probably more closely related to Is 16840 than Is 14982 is. Also in field surveys, eight species testing positive for Is 14982 were negative for Is 16840. The particle size from electron microscopic observation of leaf dip preparations was in the range reported from potyviruses, and this was confirmed by the positive serological reaction to known potyviruses. A complex of potyviruses are known to infect grasses (Brunt et al., 1999; Morales, 1994). However, there is no general agreement on the classification of these. Although Shukla et al., (1989) grouped the strains into four distinct viruses as later confirmed by Mckern et al., (1991), Edwardson and Christie (1991) do not view them as distinct species. Although aphids frequently colonize elephant grass, attempts to use these to transmit the virus isolates were unsuccessful. There is need for further characterization of our isolates in order to ascertain their identity.

Despite the fact that elephant grass is known to be an experimental host to a number of viruses (Brunt et al.,

Table 3. Reaction of field samples of some grass species from the ILRI forage germplasm collection to antibodies of five viruses in ACP-ELISA

GRASS SPECIES	ILRI No	Reaction In ACP-ELISA				
		Is 14982	Is 16840	JGMV	MDMV	SCMV
Brachiaria humidicola	730	+	+	+	-	-
Brachiaria humidicola	14828	+	-	-	-	-
Digitaria gazensis	13087	-	-	-	-	-
Digitaria milangiana	12886	-	-	+	-	+
Echinochloa cruspavonis	9381	+	+	+	-	-
Echinochloa haploclada	12773	+	+	+	-	-
Echinochloa haploclada	12821	+	-	+	-	-
Echinochloa haploclada	12839	+	+	+	-	-
Echinochloa haploclada	12931	-	-	-	-	-
Echinochloa pyramidalis	15728	+	-	-	-	-
Ergostos paniciformis	9659	+	-	-	-	-
Panicum staganimum	15730	+	-	-	-	-
Panicum staganimum	15731	+	-	-	-	-
Paspalum auriculatum	1087	-	-	+	-	+
Paspalum plicatulum	6576	-	-	-	-	-
Paspalum polystachyum	13903	-	-	-	-	-
Paspalum scrobiculatum	671	-	-	+	-	+
Paspalum scrobiculatum	1086	-	-	-	-	-
Paspalum scrobiculatum	12838	-	-	-	-	-
Paspalum scrobiculatum	13137	-	-	+	-	+
Paspalum scrobiculatum	13149	-	-	+	-	+
Paspalum scrobiculatum	13186	-	-	+	-	+
Paspalum scrobiculatum	13738	-	-	-	-	-
Paspalum scrobiculatum	13813	-	-	+	-	+
Paspalum scrobiculatum	13861	-	-	-	-	-
Paspalum scrobiculatum	13884	-	-	+	-	+
Paspalum scrobiculatum	13908	-	-	+	-	+
Paspalum sp.	681	+	-	+	-	+
Pennisetum ramosum	7738	+	+	+	-	-
Pennisetum ramosum	8463	+	+	+	-	-
Pennisetum ramosum	8747	+	+	+	-	-
Pennisetum ramosum	9662	+	+	+	-	-
Setaria incrassata	10542	+	+	-	-	-
Sorghum arundinaceum	17219	-	-	+	-	-
Urochloa panicodes	12835	+	-	+	+	-

*Is 14982. Is 16840, JGMV, JGMV, MDMV, SCMV = Antibodies to virus isolates from *Pennisetum purpureum and P. purepureum x P. typhoides and Johnson grass mosaic, maize dwarf mosaic* and *sugarcane mosaic viruses*, respectively.
+ Represents a positive reaction (absorbance value at least twice that of healthy control) and - represents a negative reaction.

1990), this is the second report of a natural infection of this host by viruses, the first being that of EGMV (Martins and Kitajima, 1993). Elephant grass stunt disease, which spreads in Uganda, is thought to be caused by a virus (Boonman, 1993), but the virus aetiology has not been proven experimentally.

The field survey results show that apart from the *Pennisetum* virus isolates, JGMV and SCMV were very prevalent in the area. SCMV spreads world wide (Brunt et al., 1990) and has been isolated from maize and sorghum in Ethopia (Alemu Lencho, personal communication). It is an important disease of maize in East Africa, and has been reported from South Africa. *Johnson grass mosaic virus* spreads in Australia and USA. This report is the first from Ethiopia. *Maize dwarf mosaic virus* has a limited distribution in Africa (Thottappilly et al., 1993). In our survey, it was detected only in *Urochloloa panicoides*. The virus is known to be seed-borne in maize. There is need to do seed transmission studies

in this host to ascertain that seeds for distribution do not carry the virus.

The viruses found in this study constitute a potential quarantine risk and hence the need for continuous monitoring and elimination. Materials for distribution are routinely indexed for the viruses now, and techniques of *in vitro* culture have been developed to eliminate the viruses from *Pennisetum* sp.

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