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# Evaluation of Four Species of Wild Yams, as Potential Natural Reservoirs of Potyviruses Infecting Yams Cultivated in Togo

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***Authors' contributions***

*This work was carried out in collaboration among all authors. Author KDA designed the study, did wild yam leaf and seeds samples collection, performed laboratory analyzes and prepared the first draft. Author MKA reviewed the study design, managed literature searches and read the manuscript. Author YMDG provided general guidance on the study and supervised the work. All authors read and approved the final manuscript.*

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## ABSTRACT

Yams cultivation in Togo is hampered by diseases caused by Potyviruses, mainly *Yam mosaic virus* (YMV) and *Yam mild mosaic virus* (YMMV). To understand the Potyviruses dissemination mechanism and to develop an efficient control method, the present study aims to establish the role of wild yams species as potential natural reservoirs of these pathogens. As such, Potyvirus susceptibility assessment was performed on four wild yams, *D. dumetorum*, *D. bulbifera*, *D. togoensis* and, *D. smilacifolia*, which grow spontaneously in yam fields in Togo. For this, phytosanitary surveys were carried out on yam fields and forests near yam plots, in July 2018 at the long rainy season, covering 27 localities in Maritime, Central and Plateaux regions of Togo, during which wild yam leaves were sampled for viruses identification. The leaves samples were analyzed first by ACP-ELISA test to detect Potyviruses using universal anti-potyvirus monoclonal antibodies, and then by RT-PCR test to identify YMV and YMMV, using respectively pairs of

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primers YMV1&YMV2 (196 pb) and YMV-CP-2F & YMV-UTR-1R (249 pb). Then 140 seedlings obtained from seeds of the four wild yams, were inoculated with YMV isolate 20-601/06. ACP-ELISA test revealed that only the leaves samples of *D. dumetorum* and *D. togoensis*, collected in Plateaux region, were infected by Potyviruses, with respectively 24.24% and 6.25% of incidence rate. But these samples were positive for neither YMV nor YMMV at RT-PCR test. However, after the inoculations, respectively 20% of seedlings of *D. dumetorum*, 52.5% *of D. bulbifera*, 64% *of D. togoensis,* and 3.33% *of D. smilacifolia*, were infected by YMV. This suggests a high potential of these yams, mostly *D. bulbifera* and *D. togoensis,* to become natural reservoirs for YMV, under high pressure of the viruses and their vectors. These wild yams control in and around yam fields can help limit Potyviruses infections.

*Keywords: Yams; natural reservoirs; Potyviruses; YMV; YMMV.*

## 1. INTRODUCTION

Cultivated yams (*Dioscorea* sp.) are widely grown in the five economic regions of Togo and have considerable nutritional and economic importance for the country. With an estimated annual tuber production of 786,394 tonnes, yams are the second most important tuber crop after cassava in Togo [1]. Yam crops in Togo are, however, prone to viral disease attacks including those caused by Potyviruses, mainly *Yam mosaic virus* (YMV) [2,3], the most important yam Potyvirus and the most economically damaging to yams in West Africa [4,5,6,7]. In Togo, the incidence of YMV on yams of the complex *Dioscorea cayenesis-rotundata*, varies between 40% and 63% depending on the production localities, and the incidence rate of Potyviruses in general, is estimated at 36.61% [3,8].

According to several studies, one of the effective methods for controlling viral diseases, is the elimination of contamination sources [9], in this case, the natural reservoirs of the viruses. In nature, the host plants of most Potyviruses are few and are limited to a few plant species, mainly plants of the *Dioscoreaceae* and *Solanaceae* family, concerning YMV [10].

In Togo, several wild yam species are listed in different agroecological zones. Some of these species, including *D. dumetorum*, *D. bulbifera*, *D. togoensis* and *D. smilacifolia*, which grow regularly in and around cultivated yams plots, can serve as natural reservoirs for viruses infecting cultivated yam. However, no detailed study has been done to determine the role of these yam species as factors of dissemination of Potyviruses in yam production areas of Togo.

The objective of this study was to evaluate the susceptibility of wild yam species, *D. dumetorum*, *D. bulbifera*, *D. togoensis* and *D. smilacifolia* to Potyviruses, namely YMV and YMMV (*Yam mild mosaic virus*), and thus to establish their role as potential natural reservoirs of these viruses. More specifically, the study proposes to identify the Potyviruses on these four wild yam species by ACP ELISA and RT-PCR tests and to evaluate their susceptibility to YMV by mechanical inoculation using a virulent isolate of the virus.

## 2. MATERIALS AND METHODS

### 2.1 Study Areas

The studies were conducted in three economic regions of Togo, also major yam producing areas of the country. These areas are Maritime region (6°30'0" N, 1°19'60" E), Plateaux region (7°30'0" N, 1°10'0" E) and Centrale region (9°15 "0" N, 1° 0'0 "E). The Maritime and Plateaux regions have a Sudano-Guinean climate with two rainy seasons including one long rainy season (mid-March to late July) and one small rainy season (early September to mid-November). Annual rainfall varies from 800 mm to 1,600 mm of rain. The Central region has a Sudanian-like climate with one long rainy season (May to October). The annual rainfall is between 1,200 and 1,500 mm [11].

### 2.2 Plant Material

The studies were carried out on four wild yam species, *D. dumetorum*, *D. bulbifera*, *D. togoensis,* and *D. smilacifolia*, regularly present as weeds in yam fields in Togo. A total of 136 wild yam leaves samples were collected for Potyviruses detection in the laboratory (Table 1). Also, seeds were collected from the four yam species to produce seedlings for inoculation with YMV isolate.

**Table 1. Number of wild yam leaves sampled and yam plants (accessions) harvested for seeds in three yam producing areas of Togo**

| Wild yam species | Plant material collection areas | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Number of leaves | | | | Yam accessions harvested for seeds | | | |
| | MR* | PR | CR | Total | MR | PR | CR | Total |
| *D. dumetorum* | 3 | 27 | 3 | 33 | 0 | 1 | 1 | 2 |
| *D. bulbifera* | 8 | 25 | 10 | 43 | 1 | 2 | 1 | 4 |
| *D. togoensis* | 0 | 23 | 9 | 32 | 0 | 3 | 2 | 5 |
| *D. smilacifolia* | 7 | 12 | 9 | 28 | 1 | 1 | 1 | 3 |
| **Total** | **18** | **87** | **31** | **136** | **2** | **7** | **5** | **14** |

**MR = Maritime region, PR = Plateaux region, CR = Central region*

## 2.3 Viruses Studied and Primers Used for Their Detection

Potyviruses of cultivated yams, including YMV and YMMV, were mainly studied. Two pairs of primers were used for the detection of both viruses (Table 2). Previously, Potyviruses were identified in spontaneous yam leaves using universal anti-potyvirus monoclonal antibodies (Agdia Inc.).

Primer pair YMV1 & YMV2 amplifies a portion of the CP region of the YMV genome while YMV-CP-2F & YMV-UTR-1R primer pair amplifies the 3' end including the non-coding UTR region of YMMV genome and a part of its CP gene.

## 2.4 Study Techniques

### 2.4.1 Collection of leaf samples and wild yam seeds

Wild yam leaves showing apparent symptoms of virus disease were collected during surveys carried out in three major yam production zones in Togo, covering 27 localities in 9 districts. The samples were collected in different environments, specifically in yam fields and in forests near yam plots. The yam leaves sampled were kept, on the field, in a cooler containing ice and then in the laboratory at -20ºC. Yam seeds were harvested from 14 wild yam plants in 7 districts of the three regions surveyed. Each wild yam plant harvested constituted an accession (Table 3). After harvest, the seeds were dried, shelled and stored in a refrigerator at +4ºC.

### 2.4.2 Immunological identification of potyviruses

The wild yam leaves sampled were analyzed by ACP-ELISA (Antibody coating plat - Enzyme-linked immunosorbent assay) test, using universal anti-potyvirus monoclonal antibodies (Agdia Inc.) and according to the protocol provided by Agdia Inc. The samples were prepared by grinding 0.25 g of yam leaves in 5 ml of carbonate buffer at pH 9.6, and then 100 µl of the centrifuged extract obtained was placed in each test-well of microtitration plates (Nunc plate). The plates were then incubated at room temperature for 1 hour and after three washes with 1X PBST, they were filled with the first anti-potyvirus monoclonal antibody diluted to 1/200. After incubation at room temperature, for overnight at + 4ºC, and after three successive washes, the plates were filled with conjugated antibody "goat anti-mouse" (dilution: 1/200) and incubated for 1 hour at room temperature. At the end of the incubation, and after washing, 200 µl of the extemporaneously prepared substrate (1 mg of p-para nitrophenyl phosphate per 1 ml of buffer) was deposited in each of the wells of the plates. The sealed plates were incubated in total darkness for 30 minutes at room temperature. The reactions in the wells were read using the

**Table 2. List of primers used for detection of YMV and YMMV**

| Primers | Sequences | Size of cDNA fragments | Viruses detected | Sources |
|---|---|---|---|---|
| YMV1 | 5'-TGCGGAACTCRAAAGAAC-3' | 196 pb | YMV | [12] |
| YMV2 | 5'-TGCCATCAAATCCAAACA-3' | | | |
| YMV-CP-2F | 5'-GGCACACAT GCAAATGAA AGC-3' | 249 pb | YMMV | [13] |
| YMV-UTR-1R | 5'-CACCAGTAGAGTGAACAT AG-3' | | | |

**Table 3. List of wild yam accessions harvested for YMV inoculations**

| Accessions | Yam species | District | Region* | Accessions | Yam species | District | Region |
|---|---|---|---|---|---|---|---|
| DuK2 | *D. dumetorum* | Kloto | PR | ToAn1 | *D. togoensis* | Anié | PR |
| DuBl1 | *D. dumetorum* | Blitta | CR | ToAn2 | *D. togoensis* | Anié | PR |
| BulY1 | *D. bulbifera* | Yoto | MR | ToBl1 | *D. togoensis* | Blitta | CR |
| BulK1 | *D. bulbifera* | Kloto | PR | ToBl2 | *D. togoensis* | Blitta | CR |
| BulK2 | *D. bulbifera* | Kloto | PR | SiZ1 | *D. smilacifolia* | Zio | MR |
| BulBl1 | *D. bulbifera* | Blitta | CR | SiDa1 | *D. smilacifolia* | Danyi | PR |
| ToHa1 | *D. togoensis* | Haho | PR | SiBl1 | *D. smilacifolia* | Blitta | CR |

**MR = Maritime region, PR = Plateaux region, CR = Central region*

spectrophotometer at 405 nm. On reading, wells with an OD (optical density) greater than or equal to twice the OD of the healthy control, were considered positive.

#### 2.4.3 Molecular identification of YMV and YMMV

ACP-ELISA positive leaves samples were analyzed by RT-PCR test to detect YMV and YMMV, the two most important yam-infected Potyviruses in West Africa [14,15,16]. Both viruses were detected in duplex in the leaves samples according to the analysis protocol as follows: PCR tubes (0.2 ml, Starlab) were filled with extracts (25 µl/tube) obtained by grinding 0.5 mg of yam leaves samples in 5 ml of carbonate buffer (pH 9.6) and centrifuged at +4ºC at 6,000 rpm for 5 minutes. The tubes were incubated overnight at +4ºC and then washed twice with sterilized 1X PBST containing Tween 20 (0.05%) and once with sterile diethylpyrocarbonate-treated (DEPC) water. The PCR tubes were immediately used or stored at -20ºC. Leaves taken from healthy yam plants in greenhouse served as negative controls. RT-PCR test was performed by adding to the contents of each PCR tube, 25 µl of RT- PCR reaction mixture (QIAgen one-step RT-PCR kit) containing detection primers of YMV (YMV1 & YMV2, 10 µM) and YMMV (YMV-CP-2F & YMV-UTR-1R, 1 µM). The RT-PCR reactions were carried out in Biometra thermocycler under the following conditions: one retrotranscription cycle (50 ºC for 30 minutes then 95ºC for 15 minutes), 35 cycles of PCR (denaturation: 94ºC for 1 minute, hybridization: 55ºC for 1 min, extension: 72ºC for 1 min) and 10 min of extension at 72ºC to end the cycles. At the end of the RT-PCR reactions, 12 µl of amplified cDNA of each of the PCR tubes, was mixed with 2 µl of 6X load buffer (2 mM Tris-HCl pH 8, 10 mM EDTA, 5% sucrose, 0.01% bromophenol blue). The mixtures were loaded with a standard size marker of 1 kb on agarose gel (1.2% or 1.5%) prepared in 0.5X TBE buffer (100 mM Tris Borate pH 8.3, 2 mM EDTA). The electrophoresis was carried out in 0.5 X TBE buffer for 35 min at 100 V. The agarose gel, after the electrophoresis, was coloured in Ethidium Bromide for 15 min and washed for 5 min. The electrophoretic bands were visualized on a UV trans-illuminator.

### 2.5 Evaluation of YMV Susceptibility of Wild Yams by Mechanical Inoculation

#### 2.5.1 Production of wild yam seedlings

Wild yam seeds collected were disinfected with Mancozèb and sprouted in Petri dishes on moistened filter paper. After germination, the seedlings were transplanted, at the first leaf stage, into 5 cm x10 cm plastic pots on sterilized potting soil. They were, then, stored in the greenhouse at a temperature of 25-34ºC during the day and 21-23ºC at night and relative humidity of 70-90%. Since YMV transmission by yam seeds is not yet demonstrated [17], the seedlings, thus produced, were considered healthy.

#### 2.5.2 Mechanical inoculation of wild yam seedlings

Mechanical inoculation was adopted in this study. Indeed, this method is recognized as the most effective for assessing plant susceptibility to viruses [10,18]. Yam seedlings were inoculated at the three-leaf stage. For this purpose, yam leaf fragments, containing YMV isolate 20-601/06, were ground in sodium phosphate buffer (0.03 M Sodium Phosphate pH 8.3) supplemented with 0.2% DIECA (Sodium diethyl-dithiocarbamate). The crushed yam leaves were filtered on hydrophilic cotton and then a pinch of activated charcoal was added to the collected filtrate. The leaf extract, thus prepared, was rubbed on the leaves of wild yam seedlings in the presence of carborundum. The inoculated leaves were washed with distilled water to remove excess inoculum. Ten seedlings (five seedlings in two replicates) were inoculated by yam accession.

Two yam plants, inoculated with only the inoculation buffer, served as negative controls for each treatment. The inoculated seedlings were kept safe from insects in wire cages. The yam plants were reinoculated 5 days later to ensure the infection. Five weeks after inoculation, leaves samples were taken from the yam seedlings and analyzed by RT-PCR for detection of YMV using primers YMV1 & YMV2. A total of 140 yam seedlings were inoculated.

#### 2.5.3 Observation techniques

The susceptibility of the wild yam seedlings was determined by: the incidence rates of Potyviruses, namely YMV and YMMV, the proportion of infected yam plants after the inoculations and the severity of viral symptoms induced.

The incidence of a viral disease is defined as the ratio of the number of plants infected with a given virus to the total number of plants sampled and tested [19,20]. The severity of a disease is the quantitative measure of the intensity of the symptoms caused on the plants [21]. The observation and evaluation of the susceptibility of wild yam seedlings started 15 days after inoculation and lasted for three weeks.

The severity of viral symptoms on yam plants was assessed on a scale of 1 to 5 [22] and defined as follows: 1 = no symptoms; 2 = moderate symptoms; 3 = severe symptoms; 4 = very severe symptoms; 5 = distortion, malformation of leaves or stems, stunting of plants.

## 3. RESULTS

### 3.1 Potyvirus Detection in Yam Leaves

Of the 136 yam leaves sampled and analyzed, only 10 were positive for Potyvirus, with an estimated average incidence rate of 7.35%. Depending on the yam species, the results were variable as shown in Table 4. Indeed, for *D. dumetorum*, of the 33 samples analyzed, 8 were positive; that corresponds to 24.24% of Potyvirus incidence rate for this species. Of these positive samples, three were collected from yam fields while the remaining five were collected from forests near yam crops. *In D. togoensis*, only 2 leaves samples from a yam field associated with maize tested positive, corresponding to 6.25% of the Potyvirus incidence rate. However, no leaf samples of *D. bulbifera* and *D. smilacifolia* were tested positive. All the 10 leaves samples tested positive for Potyviruses, were collected in Plateaux region. Leaves samples collected in Maritime and Central regions were all tested negative.

### 3.2 Identification of YMV and YMMV in Yam Leaves Samples

The 10 leaves samples tested positive for Potyviruses, were analyzed by RT-PCR for YMV and YMMV detection. However, none of the leaves samples of *D. dumetorum*, nor *D. togoensis*, was positive.

### 3.3 Reactions of the Wild Yam Seedlings to YMV Inoculation

Table 5 shows the reactions of the wild yam seedlings to mechanical inoculations with isolate 20-601/06 of YMV. It is noted in Table 5 that, in each of the four wild yam species, at least one of the inoculated seedlings were infected. However, the proportions of the infected plants varied according to the yam species. The highest proportions of diseased plants were observed in *D. togoensis* with 80% of plants infected for the accession ToB1, and in *D. bulbifera*, with 60% for accession BulK2. In *D. dumetorum*, 20% of yam seedlings were infected for, respectively, accessions DuK2 and DuBl1. In, *D. smilacifolia,* of the three accessions inoculated, only one seedling of accession SiBl1 was infected.

**Table 4. Potyvirus incidence rates by wild yam species and number of positive yam leaves samples by region**

| Wild yams species | Number of positive leaves Samples | | | Potyviruses incidence rate |
|---|---|---|---|---|
| | MR* | PR | CR | |
| *D. dumetorum* | 0/3 | 8/27 | 0/3 | 24.24% |
| *D. bulbifera* | 0/8 | 0/25 | 0/10 | 0% |
| *D. togoensis* | 0 | 2/23 | 0/9 | 6.25% |
| *D. smilacifolia* | 0/7 | 0/12 | 0/9 | 0% |
| **Total** | **0/18** | **10/87** | **0/31** | **7.35%** |

**MR = Maritime region, PR = Plateaux region, CR = Central region*

**Table 5. Reactions of wild yam seedlings to inoculation of isolate 20-601/06 of YMV: infected plants proportion and symptoms severity**

| Accessions | Yam species | The proportion of infected plants | Symptoms severity | Accessions | Yam species | The proportion of infected plants | Symptoms severity |
|---|---|---|---|---|---|---|---|
| DuK2 | *D. dumetorum* | 2/10 | 1.3±0.48 | ToAn1 | *D. togoensis* | 6/10 | 2.6±1.43 |
| DuBl1 | *D. dumetorum* | 2/10 | 1.2±0.42 | ToAn2 | *D. togoensis* | 5/10 | 2.2±1.17 |
| BulY1 | *D. bulbifera* | 6/10 | 2.5±1.35 | ToBl1 | *D. togoensis* | 8/10 | 3.1±0.97 |
| BulK1 | *D. bulbifera* | 4/10 | 1.6±0.84 | ToBl2 | *D. togoensis* | 6/10 | 2.6±1.32 |
| BulK2 | *D. bulbifera* | 6/10 | 2.3±1.25 | SiZ1 | *D. smilacifolia* | 0/10 | 1.00±0.0 |
| BulBl1 | *D. bulbifera* | 5/10 | 2.40±1.35 | SiDa1 | *D. smilacifolia* | 0/10 | 1.00±0.0 |
| ToHa1 | *D. togoensis* | 7/10 | 2.7±1.34 | SiBl1 | *D. smilacifolia* | 1/10 | 2.00±0.0 |

Concerning the severity of the viral disease symptoms induced, only seedlings of accession ToBI1 of *D. togoensis*, showed severe mosaic symptoms (cote>3). The majority of YMV-infected yam seedlings exhibited moderate mosaic symptoms (cote 2), although in some accessions, such as BulY1, BulK2 and ToHa1, high proportions of seedlings were infected. In *D. dumetorum* species, the severity of the symptoms developed was much lower with an average of 1.2 to 1.3.

The results of RT-PCR tests, carried out on the leaves of wild yam seedlings inoculated, revealed that all the seedlings showing symptoms of virus disease, were positive to YMV. Also, one of the seedlings of accession BulY1 of *D. bulbifera*, which showed no symptoms, was positive at the RT-PCR analyses.

Results of Table 6 show that wild yam species *D. togoensis* and *D. bulbifera* are the most susceptible to YMV inoculation with respectively 64% and 52.5% of infected plants and with an average severity of 2.64 and 2.2. On the other hand, *D. smilacifolia* was found to be the least susceptible to YMV with 3.33% infected plants and the average severity of 1.33.

## 4. DISCUSSION

During the serological tests carried out on leaves samples of the four wild yams studied, the positive reactions of samples belonging only to *D. dumetorum* and *D. togoensis* suggest that only these two yams species are susceptible to Potyviruses. Moreover, the fact that all these positive samples were collected in Plateaux region, could be explained by the very high pressure of Potyviruses and their vectors in this area. Indeed, in Togo, previous studies of yam viral diseases revealed Potyviruses incidence rates in Plateaux region as the highest in the country [8]. These results may also indicate the presence, in this area, of Potyvirus populations that have adapted to the two wild yam species. In the wild, especially in yam production areas in West Africa, it has been reported that Potyviruses, in this case, YMV and YMMV, are prone to a very high genetic variation and rapid evolution capabilities allowing them to be adapted or skirt the resistance of their host-plants [23,24]. In fact, in Togo, an interesting case of adaptation of Potyviruses was that the incidence of YMMV was estimated at 42.86% on *Dioscorea alata* but at 3.66% on the *D. cayenensis-rotundata* complex [25,8]. However, in Nigeria, the incidence rate of YMMV on *D. cayenensis-rotundata* complex was estimated at 46% and up to 80% in Ghana [26]. This demonstrates a great pathogenic diversity of these Potyviruses, depending on the localities and yam - hosts species.

The negative reactions, to YMV and YMMV detection tests, of *D. dumetorum* and *D. togoensis* leaves samples tested positive at ACP-ELISA, suggests infections of these two wild yams by other Potyviruses.

Also, *D. smilacifolia* and *D. bulbifera* leaves samples tested negative at ACP-ELISA tests, had, however, shown symptoms of virus diseases in the field. This suggests that these yam species have been infected by other viruses which could be those infecting cultivated yams in Togo. Indeed, several other virus families have been described on yams cultivated in Togo which could be sheltered by wild yams. Some of the viruses described are *Dioscorea alata virus* (DAV) on *D. alata* [25], *Dioscorea alata badnavirus* (DaBV) and *Cumber mosaic virus* on *D. alata* and *D. rotundata* [27]. Good knowledge of these viruses relationships with those infecting the four wild yams species studied would be useful to develop an effective and sustainable control strategy against viral diseases of cultivated yams.

Moreover, the infection of all the four wild yam species, after inoculation by YMV isolate, suggests a high capacity of these yam species, especially *D. bulbifera*, *D. togoensis,* and *D. dumetorum*, to be infected by this virus, and therefore to serve as its potential natural reservoirs, under conditions of high pressure of the viruses and intense activities of their vectors.

**Table 6. Reactions of the four wild yam species to inoculation of isolate 20-601/06 of YMV**

| Wild yam species | Infected seedlings percentage | Symptoms severity average | Wild yam species | Infected seedlings percentage | Symptoms severity average |
|---|---|---|---|---|---|
| *D. dumetorum* | 20% | 1.2 | *D. togoensis* | 64% | 2.6 |
| *D. bulbifera* | 52.5% | 2.2 | *D. smilacifolia* | 3.3% | 1.3 |

## 5. CONCLUSION

The present study showed that, in their natural environment, wild yam species, *D. dumetorum* and *D. togoensis*, *D. smilacifolia,* and *D. bulbifera*, commonly found as weeds in yam plantations in Maritime, Central and Plateaux regions of Togo, are not infected by Potyviruses infecting cultivated yams. However, results of mechanical inoculations with YMV isolate demonstrated that, under high pressure of Potyviruses and intense activities of their vectors, these yams can be infected and thus, become natural reservoirs for these viruses. In their natural habitats, some of these yams would be also infected by viruses not yet identified but whose knowledge, especially their relationships with other viruses described on cultivated yams, would be important for effective and sustainable control.

## COMPETING INTERESTS

Authors of this manuscript declared that they have not any financial and personal relationships with other people or organizations that could inappropriately influence (bias) their work.

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