Indexing and elimination of viruses infecting yams (*Dioscorea* spp.) for the safe movement of germplasm

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Abstract

Development and improvement of root crops, such as yam, that are mostly vegetatively propagated, strongly rely on the possibility to exchange genetic resources. In order to allow the international exchange of healthy germplasm, CIRAD set up a yam quarantine unit in Montpellier (France) in 2002. The main goal of this quarantine unit is to ensure exchange of disease-free germplasm. For that purpose, relevant diagnostic tools, essentially aiming at the detection of virus diseases, were implemented. In order to clean up the germplasm, *in vitro* cultures (nodal microcuttings and meristem tip culture) were used in association with heat treatment. Distribution of germplasm was ensured with *in vitro* plantlets. A first transfer of yams from Vanuatu and Benin with Guadeloupe was achieved to supply the breeding program of CIRAD with parental clones. Since 2004, about 50 clones (mainly *D. alata*) have been transferred after testing for 5 virus species or genuses [badnaviruses, Cucumber mosaic cucumovirus (CMV), potexviruses, Yam mild mosaic potyvirus (YMMV), Yam mosaic potyvirus (YMV)] following the rules of international germplasm transfer (FAO-IBPGR) and the Convention on Biological Diversity. A second transfer of *D. alata* from Vanuatu to Benin is in progress.

Keywords: yam, virus detection, virus elimination, thermotherapy, meristem culture

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Introduction

The genetic base of cultivated yams (*Dioscorea* spp.) is particularly narrow outside their respective zones of origin. As sexual reproduction is rare in cultivated yams, the appearance or the creation of new varieties more adapted to new needs, environmental changes and modifications of cultural practices, is very limited. High genetic erosion resulting in the disappearance of appreciated cultivars is noticed, in particular where there overpopulation is important. It is increased because of growing aggressiveness of anthracnose, the main disease affecting *D. alata*, and the recurrent presence of multiple virus infections, involving yield and quality reduction of harvested tubers, particularly on *D. trifida*, which is in strong regression in the Caribbean Islands and, to a lesser extent, on *D. rotundata* in West Africa.

For all these reasons, introductions of new germplasms are more and more crucial in most of the culture areas of yam. Exchanges of germplasms need, however, some precautions not to introduce pathogens infecting yams into safe areas. Nevertheless very few laboratories in the world are capable to carry out indexing and cleaning on yams. Although IITA (Nigeria) is probably the most experimented institution concerning this topic (Ng, 1988; Ng, 1994), international safe movements of yam varieties are unfortunately rare. For that purpose, CIRAD set up a yam quarantine unit in Montpellier (France) in 2002 in order to increase the production of healthy germplasm.

The technical guidelines for the safe movement of yam germplasm edited by FAO and IBPGR (Brunt *et al.*, 1989), list the main pathogens liable to infect cultivated yams. Nematods, beetles, mealybugs and anthracnose are easily eliminated by application of pesticides and by *in vitro* culture of yams at the beginning of the quarantine process. But, *in vitro* culture alone (from nodal cutting) does not allow virus elimination most of the time. Meristem culture (Saleil *et al.*, 1990) associated or not with

thermotherapy (Mantell *et al.*, 1980), and chemotherapy (Mantell, 1993; Malaurie, unpublished) aim at eradicating them and they are chosen according to the difficulty in eliminating the viral diseases and the simplicity of implementation.

Five viruses liable to infect tropical yams are targeted within the quarantine process:
1. *Badnavirus*: *Dioscorea* baciliform virus (DBV)
2. *Cucumovirus*: CMV
3. *Potexvirus*: *Dioscorea* latent virus (DLV)
4. *Potyvirus*: YMMV and YMV

Asymptomatic infection is frequent in yam and, so, the absence of symptoms is not sufficient to guarantee the healthy state of a plant. The development of serological tools such as ELISA, and, more recently, of nucleic acid based tools such as PCR and RT-PCR, helps in the detection and the diagnosis of viral diseases with very high sensitivity and specificity (Lebas, 2002). However, nucleic acid based tests seem to be more promising because they can be constantly improved for a better pertinence.

The aim of this paper is to describe the protocol used at CIRAD to index and to sanitize for viruses yams originating from Benin and Vanuatu. We also report the first obtaining of virus-free yams already released and distributed to tropical countries.

Materials and methods

Origin of material

The list of accessions studied is presented in Table 1.

Dormant tubers of 63 yam varieties (55 *D. alata*, 7 *D. nummularia*, 1 *D. trifida*) were received from the Vanuatu germplasm collection in July 2001. This germplasm was established during the South Pacific Yam Network project (SPYN, 2003) and conserved at the Vanuatu Agricultural Research and Technical Centre (VARTC). These varieties were chosen mainly for their good tolerance to anthracnose or for their good flowering ability.

Another set of dormant tubers of 27 local varieties (1 *D. alata*, 1 *D. praehensilis* and 25 *D. rotundata*) was received from Benin in December 2001 with the agreement of the Institut National des Recherches Agricoles du Bénin (INRAB). These varieties were chosen for their good tuber features (shape, organoleptic quality).

An additional batch (named “CIRAD collection”) of 4 *Dioscorea* spp. accessions from different origin was already available at CIRAD and was used for providing negative or positive controls in virus detection testing.

Table 1. Results of virus detection tests and cleaning of yam accessions, and availability of virus-free accessions at CIRAD

Accession name	Local name	Species	Country of origin	Name of collection	Virus(es) detected [a]	Virus detected after cleaning [b]	Virus-free accession avalaible
64	Africa	*D. trifida*	Vanuatu	Vanuatu	Badnavirus, YMMV	Badnavirus	No
VU019a	n.a.	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU024a	Tepuva	*D. alata*	Vanuatu	Vanuatu			Yes
VU026a	Dam masis	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU028a	n.a.	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU047a	Malingova	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU231a	n.a.	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU373a	Buhgi toa	*D. alata*	Vanuatu	Vanuatu			Yes
VU402a	Raranaeolo	*D. alata*	Vanuatu	Vanuatu			Yes
VU408a	Manioc	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU423a	Manlakon	*D. alata*	Vanuatu	Vanuatu			Yes
VU434a	Pili	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU443a	Viraïji	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU444a	Tamate ajuju	*D. alata*	Vanuatu	Vanuatu	YMMV	YMMV	No
VU454a	Mere	*D. alata*	Vanuatu	Vanuatu			Yes
VU472a	Tepuna	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU474a	Tumas	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU487a	Nusamu	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU491a	Riprip	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU495a	Malakula	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU497a	Ragir red	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU503a	Suk	*D. alata*	Vanuatu	Vanuatu			Yes
VU520a	Salomon	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU528a	Tacharamivar	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU534a	Naharto	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU540a	Behenzen	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU551a	Mombri	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU554a	Letslets masis	*D. alata*	Vanuatu	Vanuatu			Yes
VU556a	Red tumas	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU564a	Mendrovar	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU567a	Homb	*D. alata*	Vanuatu	Vanuatu	Badnavirus, YMMV	Badnavirus	No
VU573a	Betmel	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU579a	Letslets nambas	*D. alata*	Vanuatu	Vanuatu	Badnavirus	Badnavirus	No
VU590a	Makila	*D. alata*	Vanuatu	Vanuatu			Yes
VU603a	n.a.	*D. alata*	Vanuatu	Vanuatu			Yes
VU605a	Natevetev	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU613a	Tabaom	*D. alata*	Vanuatu	Vanuatu			Yes
VU639a	Malalaghi	*D. alata*	Vanuatu	Vanuatu			Yes
VU677a	Nowaneum	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU678a	Rostuan	*D. alata*	Vanuatu	Vanuatu	Badnavirus, YMMV	Badnavirus	No
VU679a	Nowanao	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU684a	Selemnu	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU688a	Kahut	*D. alata*	Vanuatu	Vanuatu	YMMV	YMMV	No
VU689a	Ifit	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU696a	Nonimanaka	*D. alata*	Vanuatu	Vanuatu	YMMV	YMMV	No
VU705a	Nouwigo	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes

VU706a	Rosapin	*D. alata*	Vanuatu	Vanuatu			No (no *in vitro* growth)
VU730a	Ross	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU735a	Noplon	*D. alata*	Vanuatu	Vanuatu	YMMV	YMMV	No
VU750a	Wanora,man	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU751a	Ross	*D. alata*	Vanuatu	Vanuatu			Yes
VU753a	Wanora,wo	*D. alata*	Vanuatu	Vanuatu			Yes
VU754a	Noulelcae	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU755a	Intejegan	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU757a	Narouvanu	*D. alata*	Vanuatu	Vanuatu	YMMV	YMMV	No
VU760a	Nureangda	*D. alata*	Vanuatu	Vanuatu	YMMV		Yes
VU618n	Braswaea	*D. nummularia*	Vanuatu	Vanuatu	YMMV		Yes
VU666n	Riprip,stron	*D. nummularia*	Vanuatu	Vanuatu	YMMV		Yes
mw2	-	*D. nummularia*	Vanuatu	Vanuatu			Yes
mw3	-	*D. nummularia*	Vanuatu	Vanuatu	Badnavirus, potexvirus		Yes
mw11	-	*D. nummularia*	Vanuatu	Vanuatu			Yes
mr7	-	*D. nummularia*	Vanuatu	Vanuatu	YMMV		Yes
ss	-	*D. nummularia*	Vanuatu	Vanuatu			Yes
Benin 1	Agogo	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 2	Aimon	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 3	Morokorou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 4	Danwari	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 5	Kponan	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 7	Sousou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 8	Baniouré (type wolokaba)	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 9	Baniouré (type yakoba)	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 11	Apo	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 12	-	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 13	Kinkerebou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 14	Douroubayessirou	*D. rotundata*	Benin	Benin	Badnavirus, YMV	Badnavirus, YMV	No
Benin 15	Baniapka	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 16	Dani	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 17	Singo	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 19	Kagorou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 20	Singou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 21	Nindouin	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 22	Mondji	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 23	Gnidou	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 25	Gnifogbado	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 27	Kannagnin	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 29	Ala-kodjêwé	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 30	Laboko	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 31	Kokoro lisse	*D. rotundata*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 32	-	*D. praehensilis*	Benin	Benin	Badnavirus	Badnavirus	No
Benin 33	Florido	*D. alata*	Benin	Benin			Yes
CGU 1b	Corossol	*D. rotundata*	Guadeloupe	CIRAD	Badnavirus, potexvirus, YMV	Badnavirus, potexvirus, YMV	No
Cuba 6	n.a.	*D. trifida*	Cuba	CIRAD	Badnavirus	Badnavirus	No
MP 2	n.a.	*D. trifida*	French Guyana	CIRAD	Badnavirus, YMMV, YMV	Badnavirus, YMMV	No
297	issued from true seed	*D. alata*	Guadeloupe	CIRAD			Yes

[a] At least 2 detection tests per accession were carried out as follows:

- Badnaviruses: coating-PCR (modified from Yang *et al.*, 2003)
- CMV: DAS-Elisa (commercial kit Biorad, France)
- Potexviruses: coating-RT-PCR (modified from van der Vlugt et Berendsen, 2002)
- YMMV and YMV: coating-duplex-RT-PCR (modified from Mumford et Seal, 1997)

[b] Heat treatment + meristem culture

Yam planting and sample collecting

Upon arrival in quarantine (Montpellier, France), the tuber skin was disinfected by soaking in a mixture of insecticide (1 g/l malathion), and fungicide (1.5 g/l mancozebe and 56.3 mg/l myclobutanil), and planted in pots containing peat (in October for Vanuatu material and in April for Benin material). Plants obtained from sprouted tubers, were cultured in a glasshouse maintained at a temperature of 20-35°C.

To initiate yam *in vitro* cultures, single node cuttings were taken from a 3-6 month-old well growing vine. At the same time, full developed leaves were collected from the same vine to make a first run of virus detection tests (see below). A second sampling of leaves was undertaken for negative tested plants, at least 3 months later, to make a second run of virus detection tests.

Virus detection

Health status of plant material was checked using different PCR and serological tests according to targeted viruses (see below). In all tests, water controls were used to reveal possible contaminations of buffer solutions. Healthy plant of *D. alata* (acc. 297) issued from true seed and maintained in an insect-proof glasshouse was used as negative control.

1. ELISA test for CMV detection.

CMV was detected by DAS–ELISA using a commercial kit (BioRad, France) according to the protocol described by the manufacturer. The positive control was a banana plant (*Musa acuminata*) infected with CMV.

2. PCR and RT-PCR test.

2.1. Sample preparation and coating: Samples were prepared by grinding fresh leaf using microfuge tube pestles in (1/10, w/v) carbonate coating buffer (34.87 mM $NaHCO_3$, 15 mM Na_2CO_3; pH 9.6), additionned with 2% (w/v) PVP-40 and 1% (w/v) Na_2SO_3. Crude extracts were then centrifuged at 6000g for 5 mn and 25 µl of supernatants were added to 0.2 ml PCR tubes. The tubes were incubated overnight at 4°C before being rinsed twice with PBS (0.137 M NaCl, 1.76 mM KH_2PO_4, 0.02 M Na_2HPO_4, 2.68 mM KCl; pH 7.4) containing 0.05% (v/v) Tween 20 and once with molecular grade water.

2.2. Targeted viruses:

Badnaviruses: Badnaviruses were detected by PCR using the universal badnavirus primers, BadnaFP (modified as follows: 5'-ATG CCI TTY GGI ITI AAR AAY GCI CC-3') and BadnaRP (Yang *et al.*, 2003), and HotStarTaq Master Mix kit (Qiagen, USA) according to the manufacturer's protocol. The final concentration of primers in PCR mix was 0.2 µM and annealing temperature (Ta) was 55°C. Accession MP2, a badnavirus-infected *D. trifida* yam, was used as positive control.

Potexviruses: Potexviruses were detected by RT-PCR using the universal potexvirus primers, Potex 2RC and Potex 5 (van der Vlugt and Berendsen, 2002), and OneStep RT-PCR kit (Qiagen, USA) according to the manufacturer's protocol. The final concentration of primers in RT-PCR mix was 1 µM and Ta was 51.5°C. The positive control was a potato plant (*Solanum tuberosum*) infected with Potato virus X (PVX) kindly supplied by J. Martin (LNPV-SQPDT, France).

YMMV and YMV: These two potyviruses were detected in the same test by duplex RT-PCR using OneStep RT-PCR kit (Qiagen, USA) according to the manufacturer's protocol and by adding the four primers (YMMV CP 2F, YMMV UTR 1R, YMV CP 1F and YMV UTR 1R) described by Mumford and Seal (1997) to the RT-PCR mix. The final concentration of primers in RT-PCR mix was 0.2 µM and Ta was 55°C. Accession MP2, a YMMV and YMV-infected *D. trifida* yam, was used as positive control.

2.3. PCR, RT-PCR and amplification analysis: 25 µl of PCR or RT-PCR mix was added to each tube and the thermal cycler was run for reverse transcription of RNA (30 mn at 50℃), in case of RNA virus detection, and for amplification of DNA (activation: 15 mn at 95℃; 35 cycles: 1 mn at 94℃, 1 mn at Ta, 1 mn at 72℃; fin al extension: 10 mn at 72℃). Amplified products were then electrophoresed through a 1.2% (w/v) agarose gel, stained in a solution of ethidium bromide (1 µg/ml) and visualised on UV transilluminator.

In vitro establishment and cultivation

Single nodes, about 2 cm long, were immersed into a 7% (v/v) bleach solution (9.6% active hypochlorite) containing a few drops of Tween 20, during 20 mn, and then, in the same solution diluted by 50%, for 10 mn. They were rinsed three times with sterile pure water. Explants were aseptically cultivated into test tubes (one node per test tube) on MS medium (Murashige and Skoog, 1962) modified as follows: macronutrients salts reduced by half, 30 g/l sucrose and 2 g/l activated charcoal. Cultures were grown in a growth chamber maintained at 25-27℃ and with a 12 h photoperiod.

Aseptic plantlets obtained after 2 months of *in vitro* culture were divided into single node cuttings, propagated on fresh culture medium in test tubes or in 500 ml flasks (25 nodes per flask), and subjected to the same growth conditions.

Thermotherapy and meristem culture

Virus infected plantlets were subcultured in 500 ml flasks on the same culture medium as above and placed in a thermotherapy incubator. Thermotherapy consisted in a hot-air treatment (34-36℃ by day and 32℃ by night; 12 h photoperiod) for 45-60 days until the *in vitro* plantlets reached 2 to 6 cm high. The day temperature could be slightly reduced if no growth was noticed.

Immediately after heat treatment, twenty apical meristem tips (0.5-1.0 mm long) per accession were aseptically excised with a scalpel from heat-treated *in vitro* plantlets under binocular microscope and placed onto the same culture medium in 90 mm Petri dishes (ten meristems per Petri dish). Petri dishes were then placed into the growth chamber. Two months later, small regenerated plantlets were transferred into test tube on fresh culture medium for further growth. *In vitro* plantlets were then cloned for *in vitro* conservation or for acclimation.

Acclimation

In vitro plantlets regenerated from meristem tip culture were removed from test tubes and their roots were briefly washed in tap water. Plantlets were then potted in a 1:2:1 peat:sand:vermiculite mixture and transferred into mini-glasshouses to maintain high relative hygrometry (80-90%) for 3 weeks. Mini-glasshouses were kept in growth chamber (28℃; 80% relative hygrometry; 12 h photoperiod). Plants were watered daily with 0.5 g/l NPK (14-12-15) fertilizer and regularly transferred into larger pots until they reached about 50 cm high and then transferred into an insect-proof glasshouse (23-35℃) for further growth.

Plants were then indexed twice for virus(es) (see above) with an interval of at least 3 months between the two tests. Plant tested positive were discarded and plants tested negative were conserved *in vitro* or in insect-proof glasshouse for future dissemination.

Results and discussion

Screening for viruses

Five different viruses from 4 different genuses were searched by Elisa and PCR tests in 90 yam accessions originating from 2 countries of 2 different continents, Benin and Vanuatu. The results of these screenings are presented in tables 1 and 2. The most common viruses found in the tested accessions are YMMV and badnaviruses, but the distribution of these viruses depends on the geographical origin and on the type of yam species. Thus, about 70% of *D. alata* yams from Vanuatu were found infected with YMMV and five (7.9%) accessions were found infected with badnaviruses. All

D. rotundata from Benin were found infected with badnaviruses. Two *D. rotundata* accessions (acc. Benin 14 and CGU 1b) and one *D. trifida* accession (acc. MP2) were also found infected with YMV. Potexviruses were found in one *D. nummularia* accession (acc. mw3) and in one *D. rotundata* accession (acc. CGU 1b). No plant positive for CMV was found in all the accessions tested. Mixed infections combining badnaviruses with YMMV, YMV or potexviruses, were also found in 7 accessions (acc. 64, VU567a, VU678a, mw3, Benin 14, CGU 1b and MP2).

Table 2. Prevalence of targeted viruses in yams depending on their origin

Targeted virus	No. of infected accessions (% analysed samples)					
	Vanuatu [a]		Benin [b]		CIRAD [c]	
Potyvirus:						
YMMV	44	(69.8)	0	(0)	1	(20.0)
YMV	0	(0)	1	(3.7)	2	(40.0)
Badnavirus:						
all strains	5	(7.9)	26	(96.3)	3	(60.0)
Potexvirus:						
all strains	1	(1.6)	0	(0)	1	(20.0)
Cucumovirus:						
CMV	0	(0)	0	(0)	0	(0)

[a] 55 *D. alata* acc., 7 *D. nummularia* acc., 1 *D. trifida* acc.
[b] 1 *D. alata* acc., 1 *D. praehensilis* acc., 25 *D. rotundata* acc.
[c] 1 *D. alata* acc., 1 *D. rotundata* acc., 2 *D. trifida* acc.

Some virus-like symptoms (mosaic, vein-chlorosis, leaf distortion and shoe-string) were observed on some of the yam plants grown in the glasshouse, but no clear correlation was established between detected virus and symptom type. Conversely, some plants with no symptom were found positive for virus, suggesting that symptom observations are not sufficient to screen for virus.

Commercial antisera for serological detection of yam viruses, excepted CMV, are not available. Therefore, immunological assays cannot be used for their detection. Fortunately, genus specific (universal) or yam virus specific PCR tests are available and we used them for virus detection in a quarantine strategy. Yam potyviruses (YMMV and YMV) are well known and specific pairs of primers were defined (Mumford and Seal, 1997; Bousalem *et al.*, 2000; Bousalem *et al.*, 2003). But, for DBV and DLV detection, no specific PCR tests are available since no recent studies were done on these viruses. Thus, we used genus specific tests (van der Vlugt and Berendsen, 2002; Yang *et al.*, 2003). But, since these tests are of broad spectrum, the exact nature of the detected viruses is not known and it is not possible to say if we detected DLV and DBV respectively or others uncharacterised strains.

We found that YMMV infects most of the Vanuatu yam accessions including *D. alata*, *D. nummularia* and *D. trifida*. Badnaviruses are also present in a lesser extent. Lebas (2002) found similar results confirming that YMMV was the main viral disease of yams in Vanuatu. Concerning the *D. rotundata* accessions, we found all of them positive for badnaviruses with the genus specific PCR test developed by Yang *et al.* (2003). These results are very similar to those found by S. Seal (unpublished) in *D. rotundata* from Guinea and by Durand (2004) in *D. rotundata* from Guadeloupe. Such a proportion of badnavirus positive plants is disturbing. Therefore, these authors, like Lebas (2002), with the help of phylogenetic analysis of some amplified sequences, suggest that badnavirus-like sequences could be integrated into *D. rotundata* genome as was already described in *Musa* spp. with Banana streak virus (BSV)(Harper *et al.*, 1999) and in taro with Taro baciliform virus (TaBV)(Yang *et al.*, 2003). If this finding is confirmed, the diagnosis method used for the badnavirus detection in yam must be called into question since no distinction could be done between integrated sequence and viral particles. Therefore, further investigations should be carried out to develop new badnavirus indexing techniques.

In vitro establishment and cultivation

On average, approximately 60 % of the node cuttings survived and regenerated in plantlets after *in vitro* establishment. Other nodes died or were not disinfected. Therefore, 6 node cuttings are enough, in general, to establish an accession *in vitro*. Excepted for one *D. alata* accession (acc. VU706a) for

which the *in vitro* culture failed because of a very poor growth, all other accessions (93 acc.) were well established *in vitro* and subcultured despite some noticeable growth speed differences between clones.

In vitro establishment of yam accessions were first attempted with meristem tip excised from mother plants grown in glasshouse and cultured on aseptic culture medium as described by Mantell *et al.* (1980) and Lebas (2002), but no growth was observed (data not shown). Additionnaly, the number of apical meristem tips is very limited on mother plants. Therefore, we decided to propagate first the accessions by *in vitro* culture of nodes before excising meristem tips for virus elimination, as Saleil *et al.* (1990).

Virus elimination

After a 2 months culture, about 35% of meristems regenerated into plantlets (table 3) and all the accessions regenerated at least one plantlet. On *D. trifida*, regeneration ratio (14.6%) is smaller than *D. alata* and *D. rotundata* accessions but only 3 accessions were tested. In preliminary tests (data not shown), we noticed that addition of activated charcoal to culture medium strongly increased the regeneration ratio particularly on *D. alata* accessions for which high quantities of toxic phenolic compounds were released in the medium. Reduction by half of the macronutrients salts on MS medium was also found beneficial for improvement of regeneration.

Table 3. Regeneration efficiency of meristem culture after heat treatment for different species of yams

Species	No. accessions	No. of cultivated meristems	No. of regenerated plantlets	Regeneration ratio (%)
D. alata	41	820	325	39.6
D. nummularia	4	140	28	20.1
D. praehensilis	1	20	17	84.6
D. rotundata	26	520	174	33.5
D. trifida	3	60	9	14.6
Total	75	1560	553	35.4

Efficiency of heat treatment combined with meristem culture on badnaviruses, potexviruses, YMMV and YMV elimination are presented in table 4. YMMV elimination was obtained on 110 plants among 238 regenerated plantlets from meristem. This good ratio (46.3%) allowed us to clean 40 accessions among the 45 YMMV-infected accessions. On the other hand, none of the 26 *D. rotundata* accessions tested for badnaviruses was PCR negative. Elimination of badnaviruses was obtained for only one *D. nummularia* accession (acc. mw3) among the 34 badnavirus-infected accessions treated. Concerning potexviruses, one (acc. mw3) of the 2 infected accessions was cleaned. One (acc. MP2) of the 3 YMV-infected accessions was also cleaned.

Table 4. Efficiency of meristem culture after heat treatment for viruses elimination in yams

Virus	Plants			Accessions [a]		
	No. regenerated	No. cleaned	Virus elimination (%)	No. regenerated	No. cleaned	Virus elimination (%)
Badnaviruses (all strains)	148	3	2.0	34 [b]	1	2.9
Potexviruses (all strains)	5	3	60.0	2 [c]	1	50.0
YMMV	238	110	46.3	45 [d]	40	88.9
YMV	14	3	21.4	3 [e]	1	33.3

[a] Each accession is represented by 1 to 6 plants
[b] 3 *D. alata* acc., 1 *D. nummularia* acc., 1 *D. praehensilis* acc., 26 *D. rotundata* acc., 3 *D. trifida* acc.
[c] 1 *D. nummularia* acc., 1 *D. rotundata* acc.
[d] 40 *D. alata* acc., 3 *D. nummularia* acc., 2 *D. trifida* acc.
[e] 2 *D. rotundata* acc., 1 *D. trifida* acc.

Well developed plants grown in glasshouse and tested negative were also controlled visually for viral symptoms. No typical symptom was observed which reinforces us in the opinion that the plants were cleaned from the virus diseases.

The use of heat treatment combined with meristem culture leads to the elimination of badnaviruses, potexviruses and potyviruses (YMMV and YMV) in several infected yams. Apical meristem tips are known to be virus-free but, because they are very small, excision of true meristems and their growth in culture media are not always satisfactory. On the other hand, heat treatment is known to eliminate certain viruses by inactivation or by inhibition of their multiplication (Mink *et al.;* 1998). In addition, using thermotherapy before meristem culture can increase the efficiency of virus elimination. We showed that this method is particularly efficient for the YMMV elimination and must be confirmed for YMV and potexviruses elimination. However, badnaviruses elimination seems to be problematic on *D. rotundata* with our protocol, especially if badnavirus-like sequences are integrated in yam genome. Therefore, no *D. rotundata* accession can be until now considered free of badnavirus. However, other cleaning methods must be tested in order to eradicate badnaviruses in case of a non yam genome integration of badnavirus-like sequences, and to clean other virus-infected accessions non curable with our technique. Among them, the use of antiviral substances (chemotherapy) allowed the elimination of some potyviruses on yam (Mantell, 1993; Malaurie, unpublished) and potato (Nascimento *et al*., 2003), and the elimination of BSV, a badnavirus-related virus, on banana (Helliot *et al*., 2003). Cryotherapy consisting in the freezing of shoot apices in liquid nitrogen is also a promising technique and allowed in particular CMV and BSV eradication on banana (Helliot *et al*., 2002).

Distribution of material

Virus-free accessions were maintained *in vitro* or in insect-proof glasshouse. The distribution of these accessions was carried out by sending *in vitro* plantlets. Since 2004, about 50 accessions have been sent to 5 countries for scientific (genetics, cell biology) or agronomical purposes (table 5).

Table 5. Safe distribution of yam accessions in different countries since 2004

Country	Institution	No. of yam accessions sent	Destination
Belgium	Université de Liège	3	Cell biology
	Université de Gembloux	12	Cell biology
Benin	CIRAD-IITA	28	Agricultural exploitation
Fiji	SPC-RGC	38	*In vitro* conservation and agricultural exploitation
Guadeloupe	CIRAD	53	Genetic, breeding and agricultural exploitation
Madagascar	Université d'Antananarivo	3	Agricultural exploitation

Forty seven *D. alata* and 6 *D. nummularia* accessions were transferred from Vanuatu and Benin with Guadeloupe between 2004 and 2006 for direct cultivation of selected varieties and to supply the breeding program of CIRAD with parental clones. After 2 years of cultivation and selection in field, some varieties were found promising (good shape, tolerance to anthracnose) and will be soon tested on farms for agricultural exploitation. Some other varieties produced flowers and seeds after controlled or open pollination. These seeds will be sown in order to develop new varieties (G. Arnau, personal communication).

Another transfer of 28 *D. alata* varieties (origin: Vanuatu) to Benin (CIRAD-IITA) is in progress for direct agronomical valorization after field selection and for the future distribution of the elite selected clones in West Africa.

Thirty eight virus-free *D. alata* varieties from Vanuatu were sent to Fiji (SPC-RGC) in 2006 for *in vitro* conservation and for future distribution within South Pacific Islands for agricultural exploitation (M. Taylor, pers. comm.) according to the South Pacific Yam Network project (SPYN, 2003), or, if needed, elsewhere in the world.

Conclusions and future work

In this paper, we reported the indexing of yam germplams for viruses and the use of heat treatment combined with meristem culture for the elimination of badnaviruses, potexviruses and potyviruses (YMMV and YMV). Our work led to the production, the certification and the distribution of healthy yam germplasms (mainly *D. alata*) in several countries. Other introductions in quarantine of new germplams, such as traditional varieties and new varieties issued from breeding programs, and other distributions of accessions are considered in the close future in order to increase the genetic diversity of local yams varieties.

On the other hand, we found all *D. rotundata* accessions positive tested with badnaviruses. Therefore, no germplasm exchange is unfortunately possible at the present day for this species without an efficient detection test able to differentiate between viral particles and supposed integrated badnavirus-like sequences and without an efficient cleaning technique available for badnaviruses elimination. More investigations in this way are now considered as crucial for the safe distribution of *D. rotundata* varieties.

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