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Exploring storage protocols for yam (Dioscorea spp.) pollen genebanking

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Abstract

Implementation of pollen genebanks allows the conservation of plant genetic resources at the haploid level, pollen genetic manipulations, scheduling of hybrid seed production, and improvement of breeding efficiency. To establish pollen storage protocols for various genotypes of West African yams, laboratory and field experiments were conducted on fresh pollen and pollen stored under various conditions at the Genetic Resources Unit International Institute of Tropical Agriculture Ibadan, Nigeria (IITA). The storage treatments examined were air-dried storage, freeze-drying followed by storage in liquid nitrogen, and hermetic cold storage without previous drying ("wet-cold" storage). Pollen maintained under dry conditions (dry-air and freeze-dried) maintained aceto-carmine stainability up to 400 days but drastically lost germination capacity, most notably under dry-air storage. But pollen samples maintained at "wet-cold" conditions under -80°C retained germination capacity after 2 years. Hand pollination with pollen of *D. rotundata* that was "wet-frozen" under -80°C for 365 days gave 69.5% fruit set and 50% fruit set after 730 days in storage. The results provide evidence to suggest recalcitrance of yam pollen grains in view of poor pollen survival with drying. Therefore, the "wet-cold" storage procedure appears to be the most promising method for the sustainable implementation of yam pollen genebanks. Pollen storage protocols for active and base collections were proposed, based on these findings.

Key words: Yams, pollen genebank, preservation protocols

Introduction

The improvement of yam through conventional breeding is constrained by complicated flowering biology characterized by dioecy, unsynchronized male and female flowering, and poor and irregular flower production which altogether limit wide hybridization (Akoroda 1981, Asiedu et al 1992). A way to overcome these barriers is by combining flowering induction with pollen storage. There are reports of the successful induction of flowering in different types of yam through agronomic manipulation, for example, staggered planting (Bai and Jos 1986) and siting crossing blocks at locations conducive for flowering (Abraham and Nair 1990, Asiedu et al 1992). Pollen storage has not been effectively implemented.

Pollen storage is important for germplasm conservation, exchanges and handling, and it enhance improved breeding efficiency (Hanna 1994). The application of pollen storage as an integrated method for the long-term conservation of yam genetic resources and for use in yam improvement programs holds great promise. First, it will allow pollen to be available throughout the breeding period, thus enhancing wide and controlled hybridization. Secondly, it will remove the constraint to wide hybridization from fluctuations in flowering in genotypes that flower irregularly from season to season. It will also circumvent the limitations to yam hybridization of unsynchronized male and female flowering by making pollen available whenever the stigma is receptive. Moreover, it will permit crosses across geographical locations. Furthermore, pollen storage will enhance conservation of the haploid level gene pool as a valuable resource base for biotechnological manipulations in yam improvement.

Storage of pollen under low temperatures as a means of plant genetic resources conservation has been widely discussed by many authors for various species (Harrington 1970, Bajaj 1987, Rajasekharan et al 1994, Kozlowski and Pallardy 2002). For yam, Akoroda (1981, 1983) reported about 5% viability in pollen lots of Guinea yam after 14 months of storage under dry-cold conditions (5 °C over-concentrated sulphuric acid). But Daniel (1997) and Daniel et al (2002) concluded that the maintenance of pollen

grains of yam would be better at high moisture levels, arguing that the potential fertility of pollen grains is highest at shedding and, at this stage, yam pollen grains are sticky (Ngu 1991), indicating a high moisture content. This paper examines potential protocols for sustainable pollen storage based on results from various experiments conducted on the pollen grains of different West African yam genotypes.

Materials and Methods

Freshly opened flowers containing mature but unshed pollen grains were collected during yam flowering seasons of 1994 through to 1996 from the germplasm maintenance and nature conservation sites of International Institute of Tropical Agriculture Ibadan, Nigeria (7 °30N', 3 °54¹E).

Flowers were collected between 9.00am and 11.00am and brought to the Seed Laboratory of Genetic Resources Unit, IITA. Collection was done by severing spikes with mature flower buds from mother plants with scissors. Anthers were scooped out from recently opened flowers (mature flower buds) with a microscope mounting needle and placed on slides smeared with a drop of aceto-carmine stain. Pollen grains were teased out of the anthers with blunt mounting needles and pollen morphological observations were done with a Labourlux™ compound light microscope.

Pollen physiology studies. Laboratory pollen viability and fertility were evaluated by aceto-carmine stainability and pollen germination capacity *in vitro*. Anthers with clusters of sticky pollen grains were placed on slightly modified Brewbaker and Kwack (BK) (1963) culture medium and incubated under room conditions for 3 hr. The modified medium consisted of 10% sucrose, 100 ppm boric acid, 300 ppm calcium nitrate, 200 ppm magnesium sulphate, 100 ppm potassium nitrate, and 7% bacto-agar in deionized water. Percentage pollen germination was estimated and tube length was measured in micrometers (µm) using the microscopic scale. An estimate of pollen fertility was calculated as a product of percentage pollen germination and the tube length. These values were log transformed to derive a pollen fertility index (PFI).

All data were collected from three different microscopic fields, each field observation representing a replicate. Mean values of percentage viability, tube length, and the estimated PFI were calculated.

Pollen storage trials

Air-dried storage. In this trial, freshly excised anthers in male flower buds were collected in vials. Each vial contained at least 15 anthers. The vials containing the anthers were left uncapped and placed in a cold room at 5 °C with a relative humidity of about 35% for 2 days. The anthers containing the pollen were then packed and sealed in an aluminium envelope containing 1g of silica gel. The sealed envelopes were stored at -80 °C, -20 °C, 5 °C, and 15 °C. Pollen samples were taken out from each storage treatment for assessment of viability and stainability at 10day intervals for the first 100 days and at 100 day intervals thereafter.

Freeze-drying and storage in liquid nitrogen. Freshly sampled male flower buds of *D. rotundata* accessions TDr 3577, TDr 3370, and TDr 1766, were freeze-dried in a Ohaus™ lyophilizer set at −60 °C and 50mm Hg vacuum for 24 hr. The viability of the pollen grains was evaluated immediately after freeze-drying. The freeze-dried male flower buds were placed in cryo-vials and plunged into liquid nitrogen in Dewar™ flasks for 1 hr. Viability of the pollen was assessed after the vials were allowed to warm and thaw at room temperature.

"Wet-cold" storage. Male flower buds containing mature anthers were placed either in cellophane packs and heat sealed or in glass vials and sealed with film tapes without drying. The sealed packs and vials were immediately stored under four cold storage conditions, 15 ± 2 °C, 5 ± 2 °C (active collection cold stores), –20 °C (base collection cold store) and −80 °C (freezing in ultra-low temperature freezer). The stored pollen of *D. rotundata* (TDr 1424) and *D. praehensilis* was examined for laboratory viability and fertility after 100 and 700 days. Fruit setting capacity during pollination with fresh and stored pollen was also investigated using pollen of *D. rotundata* accessions TDr 199, TDr 3303, TDr 3577, and TDr 3605 after 365 and 730 days of storage.

Pollen storage trials were initiated during the 1994 and 1995 cropping seasons. All the materials that were air-dried or freeze-dried were from the 1994 pollen collections.

Hand pollination trials. Unripe female flower buds were covered with thrips-proof cloth bags, tightened at the base of the spike to avoid insect pollination. After 5 days when the pistil matured, the most recently opened flowers (receptive female flowers) on the spikes were pollinated by placing test pollen grains (from fresh or stored anthers) on the stigma inside the flowers. Flowers not pollinated on a spike were removed and the spikes were re-bagged. The pollinated flowers were observed for fruit setting after 8 days. Swelling of fruit indicated successful fertilization and percentage fruit set was estimated as the number of swollen fruits per total number of pollinated flowers.

Hand pollination trials were conducted at the yam breeding plots of IITA, in 1995 (365 days of storage) and in 1996 (730 days of storage) using only test pollen of pollen lots maintained under the "wet-cold" storage method to compare results with freshly harvested pollen. The trials involved only *D. rotundata* accessions which represent the bulk of the yam improvement programme at IITA. In 1995, pollen of TDr 3577 frozen under –80 °C was used to pollinate female line 90/00/308. Fresh pollen samples from accession TDr 199 were used to pollinate as control. In 1996, the same pollen lot (TDr 3577 frozen under –80 °C) was used to cross female line 93-1 and compared with fresh pollen samples of TDr 3577, TDr 3303, and TDr 3605 used on female line 3099.

Statistical procedure. Data collected on pollen physiological characteristics were subjected to One-way ANOVA on the effects of genotype on the pollen fertility variables. Means of pollen viability and fertility variables were estimated for pollen lots. Pollen lots with the highest mean values of the various genotypes were used in the analysis and mean comparison among species was done by estimates of Least Significant Differences (LSD) at p=0.05 and the Duncan Multiple Range Tests (DMRT).

The analysis of pollen viability and fertility data collected during the pollen storage trials were subjected to factorial ANOVA on the effects of genotype and storage treatments on arc-sine equivalents of percentage *in vitro* germination. Means of pollen germination and fertility under the different storage treatments were compared with control (fresh pollen) using the DMRT letters generated by the ANOVA. Percentage germination data were transformed with the arc-sine transformation table in Gomez and Gomez (1984). The ANOVA procedure was performed on Statview™ statistical package.

Results and Discussion

Yam pollen sizes ranged from 0.25 µm diameter in *D. bulbifera* and *D. dumetorum* to 0.70 µm diameter in *D. preussii* (Table 1). Yam pollen grains were mostly round or oval in shape.

Laboratory viability and fertility assays of fresh yam pollen revealed wide variations in the *in vitro* germination capacity among the various genotypes but the PFI estimates varied narrowly. *D. praehensilis* consistently had the highest percentage pollen viability in the 1994 trial and the highest pollen fertility in the 1996 trial (Table 2). All genotypes examined through the staining test stained aceto-carmine red, implying biochemical viability. Functional viability shown by the percentage germination capacity was over 30% for all the species and PFI estimates ranged between 4 and 6, except for the exotic *D. bulbifera* accessions that appeared to be sterile.

The results showing high pollen germination capacity from the various viability tests conducted on the pollen of various West African yam confirmed that yam pollen grains are naturally viable, (Akoroda 1983). The viability of freshly harvested pollen as the *in vitro* germination and aceto-carmine stainability tests is an indication of the high potential viability necessary for the successful implementation of pollen banks, since genebanking is about maintaining the materials in living and functional condition. The result suggest that the poor seed setting commonly reported in West African yam (Doku 1978, Hanson 1986, Okoli 1991) is not as a result of poor pollen viability, but is probably evidence of the poor efficiency of natural pollinators, i.e., thrips, or of non-synchronized male-female flowering. This further intensifies the potential benefits for establishing and maintaining yam pollen genebanks.

Results from the dry air storage trial are shown (Table 3). Dry air storage under -80, 5, and 15 °C resulted in a total loss of germination capacity and acetocarmine stainability in all yam pollen. But after air-dry storage of yam anthers inside -20 °C cold stores, some pollen lots maintained over 40% stainability in aceto-carmine after 30 and 400 days of storage but had totally lost *in vitro* germination capacity at both periods (Table 3).

The pollen grains of all the genotypes of yam subjected to the freeze-drying procedure maintained very high percentages of pollen stainability, but percentage germination capacity was lost in all genotypes except *D. alata* that still maintained 15% germination capacity (Table 4). The procedure for freeze-drying in the experiments appeared to have detrimental effects on most of the yam pollen grains.

Both dry storage procedures resulted in high pollen stainability but very poor *in vitro* germination capacity in most of the species, indicating the activity of certain enzymes. Impairment of germination, might be due to the low moisture availability for the process. Previous reports on pollen storage of various yam showed the same trend of low germination capacities in response to dry-storage treatments (Akoroda 1983, Ng and Daniel 2000). Hong et al 1999 reported that the longevity of *Typha latifolia* pollen improved with dry and low temperature storage, indicating "orthodox" storage behaviour. These results suggest that yam pollen grains exhibit some degree of recalcitrance, that is, yam pollen, though viable, require certain level of moisture content to germinate. Connor and Towill (1993) proposed the need to determine optimum

moisture levels at which to maintain viability at low temperatures for different pollen species instead of a general routine desiccation. There is, therefore, a research gap especially in yam pollen drying procedures to investigate the threshold moisture levels of dried flower buds that permit pollen germination, before implementation for practical pollen storage.

Table 5 shows pollen viability of two yam species after 100 and 730 days of "wet-cold" storage. At 100 days of storage, germination capacity was maintained in pollen lots stored under 5, -20, -80°C, but viability was totally lost in pollen of both species refrigerated at 15°C. Pollen frozen under -80 and -20°C germinated comparably well with fresh pollen at 100 and 730 days. A comparison of pollen *in vitro* germination data at 100 days with pollen *in vitro* germination at 730 days of "wet-cold" storage showed an insignificant loss of viability in pollen frozen under -80 and -20°C between the two periods. Pollen of *D. rotundata* wet-frozen under -80°C for 365 days gave a high laboratory pollen germination and a fertility index of 2.4, corroborated by 69.5% fruit set on the field which was insignificantly different from the fruit set when fresh pollen was used (Table 6). After 730 days (2 years) of wet-freezing, the fruit set when frozen pollen was used was not quite as close to the fruit set when pollination was done with fresh pollen, even though the laboratory PFI estimates were lower in frozen pollen than in all the fresh pollen lots (Table 6).

When compared with results from dry-cold storage trials (Akoroda 1981, 1983; Ng and Daniel 2000), "wet-cold" storage appeared to be the most effective method for the preservation of pollen viability and fertility in yam. Though desiccation was recommended for the freeze preservation of plant cells and tissues (Bajaj 1987, Hughes and Lee 1991), preserving moisture in fresh yam pollen appears more beneficial than desiccation for maintaining their fertility. The pollen grains did not exhibit the expected injuries that were apparent in the surrounding tissues of the anthers after freezing and thawing. These results demonstrated the possibility of successfully preserving the viability and fertility of yam pollen grains by ultra-low temperature storage of fresh anthers or flower buds ("wet-cold" storage).

Table1. Pollen sizes in different yam genotypes

Species	Pollen diameter	Pollen shape
D. alata	0.60a	Round
D. bulbifera	0.25b	Round
D. dumetorum	0.25b	Oval
D. praehensilis	0.30b	Round
D. preussii	0.70a	Oval
D. rotundata	0.30b	Round
D. togoensis	0.30b	Round
LSD (p=0.05)	0.163	

Means in a column followed by the same letters are not significantly different at P=0.05 by the DMRT

Protocols

The aim of this paper is to suggest protocols for preserving the haploid level genetic diversity of yam in the form of pollen form under genebank conditions to supplement classic clonal preservation methods. In genebanks, seed germplasm is maintained as active and base collections in medium and long-term conditions. In active collection genebanks, materials are usually retrieved for use within a short period of time. The results of this study showed that active collections of yam pollen grains can be maintained within a breeding season for 1-3 months when stored "wet-cold" under 5 °C as shown in protocol 1 (Table 7). This is most beneficial when the problem of non-synchronization of male and female flowering is limiting breeding efficiency.

Base collections are materials to be maintained on a long-term basis under conditions of storage that induce minimal losses in viability over time. The maintenance of pollen viability under frozen conditions for 2 years in this study implies that pollen can be maintained as base collections when wet-frozen. Protocol 2 (Table 7) shows the procedure used for wet-freezing of yam pollen in ultra-low freezers. A cheaper freezing protocol that could still be investigated for yam pollen grains is the liquid nitrogen (LN) storage in flasks. Though the results of this study did not suggest that this approach effectively preserved pollen grains, several aspects of the protocol still needs to be investigated and fine-tuned. These include pollen drying methods for LN storage, application of cryostats for LN storage, and retrieval procedures that will result in improved germination capacity of the pollen of various yam genotypes.

From this study,, the "wet-cold" preservation procedure was the most promising approach to implement the establishment of pollen banks from this study. Protocols for the implementation of this method were presented in five stages of operations including collection, sorting, packaging, storage, and retrieval. When these protocols are made available to genebank managers the objectives of pollen storage for yam breeding and improvement would be achieved.

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Table 2. Viability and fertility of fresh yam pollen grains (1994 and 1996 data).

Species	1994			1996		
	% Germ.	% Stain	PFI	% Germ.	% Stain	PFI
D. alata	64.3a	22.1b	-	53.1bc	61.9b	5.7a
D. bulbifera (exotic)	0b	0c	-	0	3.8d	0
D. bulbifera (wild)	-	-	-	71.3	-	5.1a
D. dumetorum	79.66a	83.30a	-	49.1bc	79.9a	4.9ab
D. praehensilis	100a	100a	-	87.9	-	5.9a
D. preussii	-	-	-	65.7b	-	5.3ab
D. rotundata	69.89a	73.36a	-	33.1c	63.8a	4.1bc
LSD (p=0.05)	38.528	39.236		25.429	32.507	1.811

Means in a column followed by the same letters are not significantly different at P=0.05 by the DMRT

Table 3. Percentage pollen viability (germination in vitro and aceto-carmine stainability) of yam genotypes after air-drying and storage at −20°C.

Genotype Accession	Storage period (days)					
	0		30		400	
	% Germ.	% Stain	% Germ.	% Stain	% Germ.	% Stain
D. alata 0247	34.6b	93.8a	0	65.6a	0	51.3a
D. dumetorum 2788	86.7a	62.5ab	0	66.7a	0	44.7bz
D. rotundata 3577	59.0ab	50.9b	0	50.3a	0	48.9ab
LSD (p=0.05)	35.360	30.108		12.385		3.031

Means in a column followed by the same letters are not significantly different at P=0.05 by the DMRT

Table 4. Percentage pollen germination in vitro, percentage aceto-carmine stainability and fertility index of yam genotypes in response to 24 hours of freeze-drying and 1-hour storage at vapor phase liquid nitrogen

Genotype Accession	% Germ.	% Stain	PFI
D. alata 0487	15.4a	100.0a	1.8a
D. dumetorum 2788	0b	100.0a	0b
D. praehensilis (wild)	0b	100.0a	0b
D. rotundata 0276	0b	88.8b	0b
LSD (p=0.05)	9.048	6.58	1.058

Means in a column followed by same letters are not significantly different at P=0.05 by the DMRT

Table 5. Percentage in vitro germination of yam pollen after 100 days and 2 years under hermetic "wet-cold" at various temperature regimes. Data for fresh and stored pollen were analysed separately for each temperature regime.

Genotype Accession		−80°C	−20°C	5°C	15°C
D. rotundata 1424	fresh	70a	70a	70a	70a
	100 days	74a	78a	37b	18b
	730 days	56b	--*	0c	0c
D. praehensilis (wild)	fresh	100a	100a	100a	100a
	100 days	81a	62b	47b	0c
	730 days	78a	73a	0c	0c
LSD (p=0.05)		13.865	32.407	37.718	41.454

Means in a column followed by the same letters are not significantly different at P=0.05 by the DMRT

*No data due to exhaustion of pollen samples in this store

Table 6. In vitro germination, laboratory fertility, and fruit setting capacity of fresh and wet-frozen yam pollen at –80°C. (Female parent was line 90/00/308 on IITA's breeding plot in the first year trial; female parents 93-1 and 3099 were used in the second year trial and data for each year were analysed separately).

Pollen genotype 1 year	% Germination	Pollen tube length (µm)	Fertility index (log)	% fruit setting
Fresh pollen				
D. rotundata 0199	56.0a	2.2a	2.1a	80.0a
Frozen pollen (−80 °C)				
D. rotundata 3577	75.8a	3.3a	2.4a	69.5a
LSD (p=0.05)	23.265	1.293	0.352	12.338
2 years				
Fresh pollen				
D. rotundata 3577	73.0a	2.75a	2.3a	68.0a
D. rotundata 3303	60.0a	2.75a	2.2a	85.0a
D. rotundata 3605	68.0a	2.15a	2.2a	50.0b
Frozen pollen (−80 °C)				
D. rotundata 3577	40.0b	1.50b	1.8b	50.0b
LSD (p=0.05)	17.061	0.703	0.261	19.740

Means in a column followed by the same letters are not significantly different at P=0.05 by the DMRT

Table 7. Pollen storage protocols for implementation in genebanks.

Operation	Protocol 1 Active collection	Protocol 2 Base collection
Collection	i) Cut freshly opened flowers between 9.00 and 11.00 am	i) Cut freshly opened flowers between 9.00 and 11.00 am
Sorting and Packaging	i) Conduct laboratory pollen viability assessments (in-vitro) germination and aceto-carmine staining tests) on excised anthers from randomly sampled flowers ii) Discard flowers and anthers with poor pollen viability iii) Excise anthers from flower buds iv) Place in glass vials v) Seal with parafilm and label	i) Package fresh flowers in polythene pouches ii) Heat seal pouches and label
Storage	Place sealed glass vials under 5 °C cold store or refrigerator	Place packed polythene pouches in ultra-low freezers (-80°C or -20°C)
Retrieval	Draw from store and pollinate directly on dry ice throughout transit.	i) Draw pouches from store ii) Thaw on dry ice for at least 2 hours in lab when pollination* plot is nearby. For distant pollination, place pouches iii) Thaw at field condition for at least 20 min before pollination iv) Excise anthers from flower buds to pollinate thereafter.

*Pollinate only open flower buds in the morning hours

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