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

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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 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. 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 (*Dioscorea* spp.) 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 enhances 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. Firstly,

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 bio-technological manipu-lations 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)

Plate 1. Germination and acetocarmine staining of pollen grains of *D. rotundata* in sucrose bacto-agar media. Tube length was measured with the ocular scale of the microscopic lens. Pollen fertility index (PFI) was calculated as product of *in vitro* pollen germination counts and tube length.

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 the International Institute of Tropical Agriculture Ibadan, Nigeria (7 °30¹N, 3 °54¹E).

Flowers were collected between 9.00 am and 11.00 am 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.

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 h. 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 deionised water. Percentage pollen germination was estimated and tube length was measured in micrometers (μm) using the ocular scale of the microscopic lens (Plate 1). An estimate of pollen fertility was calculated as a product of percentage pollen germination and 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 1 g of silica gel. The sealed envelopes were stored at –80, –20, 5 and 15°C. Pollen samples were taken out from each storage treatment for assessment of viability and stainability at 10-day 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 50 mm Hg vacuum for 24 h. The viability of the pollen grains were evaluated immediately after freeze-drying. The freeze-dried male flower buds were placed in

Table 1. 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.

cryovials and plunged into liquid nitrogen in Dewar™ flasks for 1 h. 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. 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.

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

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 1). 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 suggested 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, that is, 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 in Table 2. 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 2).

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 3). The procedure for freeze-drying in the experiments appeared to have detrimental effects on most of the yam

Table 2. 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	44.7b
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 3. Percentage pollen germination *in vitro*, percentage aceto-carmine stainability and fertility index of yam genotypes in response to 24 h of freeze-drying and 1 h 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.

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 yams 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 4 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.

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).

Protocols

The aim of this paper is to suggest protocols for preserving the haploid level genetic diversity of yam in the form of pollen 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

Table 4. 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 analyzed 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 were not significantly different at P = 0.05 by the DMRT. *No data due to exhaustion of pollen samples in this store.

Table 5. Pollen storage protocols for implementation in genebanks.

Operation	Protocol 1	Protocol 2
	Active collection	**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 with low 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 or –20°C)
Retrieval	Draw from store and pollinate directly	i) Draw pouches from store ii) Thaw on dry ice for at least 2 h in lab when pollination* plot is nearby. For distant pollination, place pouches on dry ice throughout transit. iii) Thaw at field condition for at least 20 min before pollination

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 5). 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 high pollen viability under frozen conditions for 2 years in this study implies that base collections of pollen grains can be preserved wet-frozen. Protocol 2 (Table 5 shows

the procedure used for wet-freezing of yam pollen in ultra-low freezers). Wet-freezing in liquid nitrogen (LN) is a protocol that could still be investigated for yam pollen grains, though the results of this study did not suggest that this approach effectively preserved pollen grains. Aspects of research important for LN storage of yam pollen are drying methods, application of cryostats and retrieval procedures that will result in improved germination capacity of the yam pollen.

From this study, the "wet-cold" preservation procedure was the most promising approach to implement the establishment of pollen banks. 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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