

Interspecific Crossing between Yam Species (*Dioscorea rotundata* and *Dioscorea bulbifera*) through *in Vitro* Ovule Culture

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Abstract

In the present study, *in vitro* ovule culture technique was used to obtain interspecific cross combination of *Dioscorea rotundata ufenyi* and *Dioscorea bulbifera wild*. Ten days after pollination, ovules were excised and cultured onto 1/2 strength Murashige and Skoog (MS) medium (Basal salt mixture + Vitamins) supplemented with 6% sucrose, 0.7% agar and plant growth hormones such as GA3, BAP, Picrolam and TDZ. Cultured ovules were transferred on 1/2 MS medium with 3% sucrose and 0.7% agar after three weeks. 40 days after pollination, germination was observed from 7 months cultured ovule between *D. rotundata ufenyi* x *D. bulbifera wild*. Hybridity of the regenerated plant was checked by flow cytometric method. A close relation was observed between the fluorescence intensity of the obtained progeny with one of the parents' fluorescence. The observed progeny can be closely correlated with an apomictic tissue from an ovule parent of *D. rotundata ufenyi*. Plantlets derived from ovule culture were proliferated through *in vitro* shoot multiplication with hormonal concentration (0.5 mg/l BAP) supplemented with 1/2 strength MS medium. Obtained ovule culture derived *in vitro* plantlets were successfully hardened, acclimatized and transferred to the field, where they survived and grew normally. In plant breeding, interspecific crossing is very important technique, enabling the time needed to produce homozygous lines to be shortened as compared to the conventional plant breeding techniques.

Keywords

Interspecific Crossing, Ovule Culture, *in Vitro*, *Dioscorea rotundata*, *Dioscorea bulbifera*

1. Introduction

Yam is cultivated as a staple food crop in Africa, Asia, Latin America, and the Caribbean. From a regional

perspective, 96% of total production of yam in the world is produced in Africa (FAO 2007). Species produced in Africa are white guinea yam (*D. rotundata* Poir), yellow guinea yam (*D. cayenensis* Lam), water yam (*D. alata* L.), bitter yam (*D. dumetorum* (Kunth) Pax.), lesser yam (*D. esculenta* (Lour) Burk.), and aerial yam (*D. bulbifera* L.). White guinea yam (*D. rotundata*), is produced mostly in Africa and most important cultivated yam with 79% of the total world food yam. *D. rotundata* is economically important due to its large edible tubers production with 15 - 20 kg weight and mostly consumed as a food choice in Nigerian traditional ceremonies. In Africa, most are pounded into a paste to make the traditional dish of “pounded yam”. *Dioscorea bulbifera*, the “air potato”, is found in both Africa and Asia and used as a folk remedy to treat conjunctivitis, diarrhea and dysentery. It is a large vine, 6 meters or more in length and produces tubers. However, the bulbils which grow at the base of its leaves are the more important food product. In West Africa, yam is an excellent source of carbohydrate energy for the people. It also includes nutrients such as vitamins, minerals and dietary protein. Yam has a high market value in Nigeria and is widely consumed in Latin America, the Caribbean, Asia, and the Pacific Islands. From a long time ago, Japanese people also have eaten raw yam as “Tororo”. It is still a common food in Japan now. Yam is a member of the family Dioscoreaceae. The genus *Dioscorea* has been reported to contain about 600 species [1].

To contribute to reducing poverty and increasing food security, IITA is breeding yam to produce new varieties based on demand and value addition. However, the breeding process is still being constrained by poor flowering, low fruit setting, a low rate of seed germination and differences in flowering periods. Interspecific hybridization is an important driving force in plant evolution and speciation [2]. Incompatibility barriers prevent interspecific crosses in hybridization between distant species [3]. Direct *in vitro* pollination of stigma or pistils and opened ovaries or ovules may be useful in overcoming incompatibility barriers [4] [5]. The best results have been achieved in species with large ovaries containing many ovules, such as those belong to Brassicaceae, Caryophyllaceae, Papaveraceae, Primulaceae and Solanaceae families [6] [7]. Kameya and Hinata [8] used ovule pollination to obtain hybrids between *Brassica* species. There are some reports about interspecific hybridization within *Dioscorea*. For example in Japan, the hybridization between *D. japonica* and *D. opposita* was produced with the aid of an embryo rescue technique [9]. However, there are no reports of interspecific hybridization between *D. alata* and *D. rotundata*, which are the most common species in Nigeria. To produce additional variations in *Dioscorea* species, interspecific hybridization of yam is required.

The main objective of this study was to develop a method for the interspecific crossing of *Dioscorea rotundata* *ufenyi* and *D. bulbifera* *wild* via *in vivo* pollination through ovule culture to produce new genotype of *Dioscorea*.

2. Materials and Methods

2.1. Plant Material, Culture Medium and Culture Initiation

In Yam species interspecific crossing *Dioscorea rotundata* *ufenyi* was used as the female (ovule) parent and *Dioscorea bulbifera* *wild* were selected as pollen parent. Parental parents were grown in IITA, Nigeria field. Closed flower buds were harvested from *Dioscorea rotundata* *ufenyi* about 48 h before opening. These flower buds were surface sterilized in laminar air flow cabinet for 30 seconds in 70% - 80% ethyl alcohol.

Enlarged ovules were excised from the ovary 10 days after *in vivo* pollination and transferred to Murashige and Skoog (MS) medium [10] supplemented with 6% sucrose, 0.7% agar and different plant growth regulators such as 6-Benzylaminopurine (BAP), Thidiazuron (TDZ), Picrolam and Gibberellic acid (GA₃). After 3 weeks, cultured ovules were transferred on 1/2 strength MS media (Basal salt mixture + Vitamins) with 3% sucrose + 0.7% agar and the ovule is opened, if necessary. The ovule derived plants were multiplied through multiple shoot differentiation with different concentrations of BAP in the 1/2 MS medium. Various concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/L) of Indole-3-acetic acid (IAA) were used for rooting. The cultures were maintained at 25°C ± 2°C temperature with 16 hours illumination with a photon flux density of 2500 lux from white fluorescent tubes (Philips, India). Cultures were transferred into new medium after every 2 - 3 weeks for best growth of plants.

2.2. Hardening and Acclimatization

Rooted shoots after one month old cultures were ready for transplanting in hardening medium. Rooted shoots were transferred to soil under shade house after *in vitro* hardening the plantlets were taken out from the flasks,

washed to remove adhered agar and then transferred to polybags containing carbonized rice husk and 2/3 of sterilized topsoil. These plantlets were supplied with half strength MS solution (without organics) twice in a week for three weeks. After three weeks, these bottles were shifted to mist chamber having relative humidity of 60% - 80% with a temperature of $34^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 3 - 4 days before they were transferred to polybags containing a mixture of carbonized rice husk and sterilized topsoil. In the mist chamber, the plants were kept for four weeks and were irrigated with half strength MS medium. Later, these polybags were shifted to tissue culture room for acclimatization before exposing to the natural environment. These obtained *in vitro* plantlets were successfully hardened, acclimatized and transferred to the field, where they exhibit normal growth.

2.3. Flow Cytometric Analysis

Relative DNA contents of nuclei isolated from leaf tissues of plantlets regenerated from ovule culture of *D. rotundata* \times *D. bulbifera* were measured using flow cytometer (PA, Partec). Samples were prepared according to Galbraith *et al.* [11]. Squeezed plant tissue containing 2 mL lysis buffer with 4',6-diamidino-2-phenylindole and β -mercaptoethanol. Filter this suspension through nylon with mesh size 30- μm and the analyses were performed using PAPII (Partec, Germany) flow cytometer. Histograms were analyzed by the use of DPAC v.2.2 software (Partec GmbH, Germany).

2.4. Statistical Analysis

Twelve replicates were used per treatment on each shooting and rooting medium. All experiments were repeated thrice. The data recorded for different parameters during the study were analyzed by using ANOVA, variation among means was compared by F-test and the critical difference (CD) values at 5% computed.

3. Results and Discussion

3.1. Interspecific Crossing by *in Vivo* Pollination and Ovule Culture

Dioscorea rotundata ufenyi and *Dioscorea bulbifera wild* were used in interspecific hybridization or crossing. In this study, 26 *in vivo* pollinated ovaries with ovules were obtained from 43 pollinated flowers after 10, 20, 30, 40 days after pollination (DAP). Several days after pollination, 153 ovules were observed. All ovules were cultured *in vitro* on 1/2 strength MS medium [10] with different growth regulators for further development. Several days after pollination, all 153 *in vivo* pollinated ovules were excised and cultured on to half-strength Murashige and Skoog (MS) media (Basal salt mixture + Vitamins) supplemented with 6% sucrose, 0.7% agar and with different concentrations of different plant growth regulators such as 6-Benzylaminopurine (1.0 mg/L BAP), Thidiazuron (0.5 mg/L TDZ), Picrolam (0.5 mg/L) and Gibberellic acid (1.0 mg/L GA₃). After 3 weeks, well grown cultured ovules were transferred on 1/2 MS media (Basal salt mixture + Vitamins) supplemented with 3% sucrose + 0.7% agar and the ovules are opened if necessary (**Figure 2(a)**, **Figure 2(b)**). Germination was observed from 7 months cultured ovule, equivalent to 40 days after pollination between *D. rotundata ufenyi* \times *D. bulbifera wild* (**Figure 2(c)**). After several weeks, the resultant germinated ovule were transferred onto 1/2 MS medium + 3% sucrose + 0.7% agar supplemented with BAP and IBA, for further multiplication and development of shoots and roots. The ovule-derived plantlets were multiplied through multiple shoot differentiation, which was markedly influenced by the concentration of growth regulator (1.0 mg/l BAP) in the 1/2 MS medium for shooting and (2.0 mg/L IAA) for rooting **Table 1**, **Figure 2(d)**. Kato *et al.* [12] recorded similar results in ornamental plants for production of interspecific hybrids. Mathiyazgahan *et al.* [13] and Ahlawati *et al.* [14] also reported similar results in guar *Cyamopsis* species. The total number of *in vivo* pollinated ovaries, total number of cultured ovules, different types of growth medium and number of germinated ovule of *D. rotundata ufenyi* \times *D. bulbifera wild* crosses are presented in **Table 2**. After *in vivo* pollination of 26 ovaries, 153 ovules were isolated, and 12 plants were recovered.

3.2. Flow Cytometric analysis, Hardening and Acclimatization

Relative DNA contents of nuclei isolated from leaf tissues of plantlets regenerated from ovule culture of *D. rotundata* \times *D. bulbifera* were measured using flow cytometry (PA, Partec). Flow cytometric analysis was used to

Table 1. Effect of plant hormones cytokinin BAP in 1/2 MS medium on shoot proliferation of *D. rotundata ufenyi* x *D. bulbifera wild*.

Plant Hormone Concentration (mg/l)	Response %	Mean shoot number	Mean shoot length (cm)
BAP			
0.0	85.25 ± 0.55	2.0 ± 0.22	4.95 ± 0.05
1.0	90.70 ± 0.40	3.7 ± 0.17	5.80 ± 0.13
2.0	68.75 ± 0.56	1.6 ± 0.21	3.98 ± 0.03
3.0	45.00 ± 0.32	1.5 ± 0.20	3.78 ± 0.02
4.0	33.33 ± 0.57	1.0 ± 0.17	2.91 ± 0.01
Effect of plant hormones auxin IAA in 1/2 MS medium on rooting of <i>D. rotundata ufenyi</i> x <i>D. bulbifera wild</i>			
IAA	Response %	Mean root number	Mean root length (cm)
0.5	46.25 ± 0.35	2.5 ± 0.15	1.38 ± 0.02
1.0	50.00 ± 0.38	3.5 ± 0.15	2.00 ± 0.03
1.5	57.20 ± 0.46	4.8 ± 0.15	3.06 ± 0.05
2.0	68.75 ± 0.56	5.5 ± 0.16	7.10 ± 0.08
2.5	65.00 ± 0.51	5.1 ± 0.15	7.78 ± 0.06
3.0	60.33 ± 0.48	4.9 ± 0.16	5.26 ± 0.04

Table 2. Total number of *in vivo* pollinated ovaries, total number of cultured ovules, different types of growth medium and number of germinated ovule of *D. rotundata ufenyi* x *D. bulbifera wild* crosses.

Crosses	Days after Pollination (DAP)	Type of pollen	No of flowers pollinated	Total number of ovary	Total number of cultured ovule	Type of medium	Germinated ovule
<i>TDr ufenyi</i> × <i>TDb Wild</i>	10	Fresh	10	8	47	BAP 1.0	0
						GA3 1.0	0
						PCR 0.5	0
						TDZ 0.5	0
	20	Fresh	10	9	54	BAP 1.0	0
						GA3 1.0	0
						PCR 0.5	0
						TDZ 0.5	0
	30	Fresh	11	-	-	BAP 1.0	0
						GA3 1.0	0
						PCR 0.5	0
						TDZ 0.5	0
40	Fresh	12	9	52	BAP 1.0	2	
					GA3 1.0	0	
					PCR 0.5	0	
					TDZ 0.5	0	

confirm hybridity of the regenerated plant [11]. The fluorescence intensity of the obtained progeny was closely related to one of the parents' fluorescence. This indicated that the observed progeny may be an apomixtic tissue from an ovule parent of *D. rotundata ufenyi*, (**Figure 1(a)**) showing histograms from flow cytometric profile of *D. rotundata ufenyi* (**Figure 1(b)**) showing histograms from flow cytometric profile of *D. bulbifera wild* (**Figure 1(c)**) plantlet between *D. rotundata ufenyi* x *D. bulbifera wild* (**Figure 1(d)**) obtained plantlet derived from ovule from *D. rotundata ufenyi* x *D. bulbifera wild*. Flow cytometry is widely used to check the hybridity of the regenerated plantlets obtained from interspecific crossing and rapid compared to the traditional karyotyping methods and the use of other morphological characteristics, especially in the analysis of generated hybrid plants.

One month old cultures rooted shoots were ready for transplanting in hardening medium. Rooted shoots were transferred to soil under shade house after *in vitro* hardening the plantlets were taken out from the flasks, washed to remove adhered agar and then transferred to polybags containing carbonized rice husk and 2/3 of sterilized topsoil. These plantlets were supplied with half strength MS solution (without organics) twice in a week for three weeks. After three weeks, these bottles were shifted to mist chamber having relative humidity of 60% - 80% with a temperature of $34^{\circ}\text{C} \pm 2^{\circ}\text{C}$. The caps of bottles were removed and plantlets were allowed to remain in the bottle for 3 - 4 days before they were transferred to polybags containing a mixture of carbonized rice husk and sterilized topsoil. In the mist chamber, the plants were kept for four weeks and were irrigated with half strength MS medium. Later, these polybags were shifted to tissue culture room for acclimatization before exposing to the natural environment. (**Figures 2(e)-(g)**) showed acclimatized *in vitro* plantlets and hardening of interspecific crosses of *Dioscorea rotundata* × *Dioscorea bulbifera*. Jovanka *et al.* [15] and Kaneko *et al.* [16] reported similar results in roles of interspecific hybridization in sunflower breeding and Brassicaceae crops. These obtained *in vitro* plantlets were successfully hardened, acclimatized and transferred to the field, where they exhibit normal growth (**Figure 2(h)** and **Figure 2(i)**).

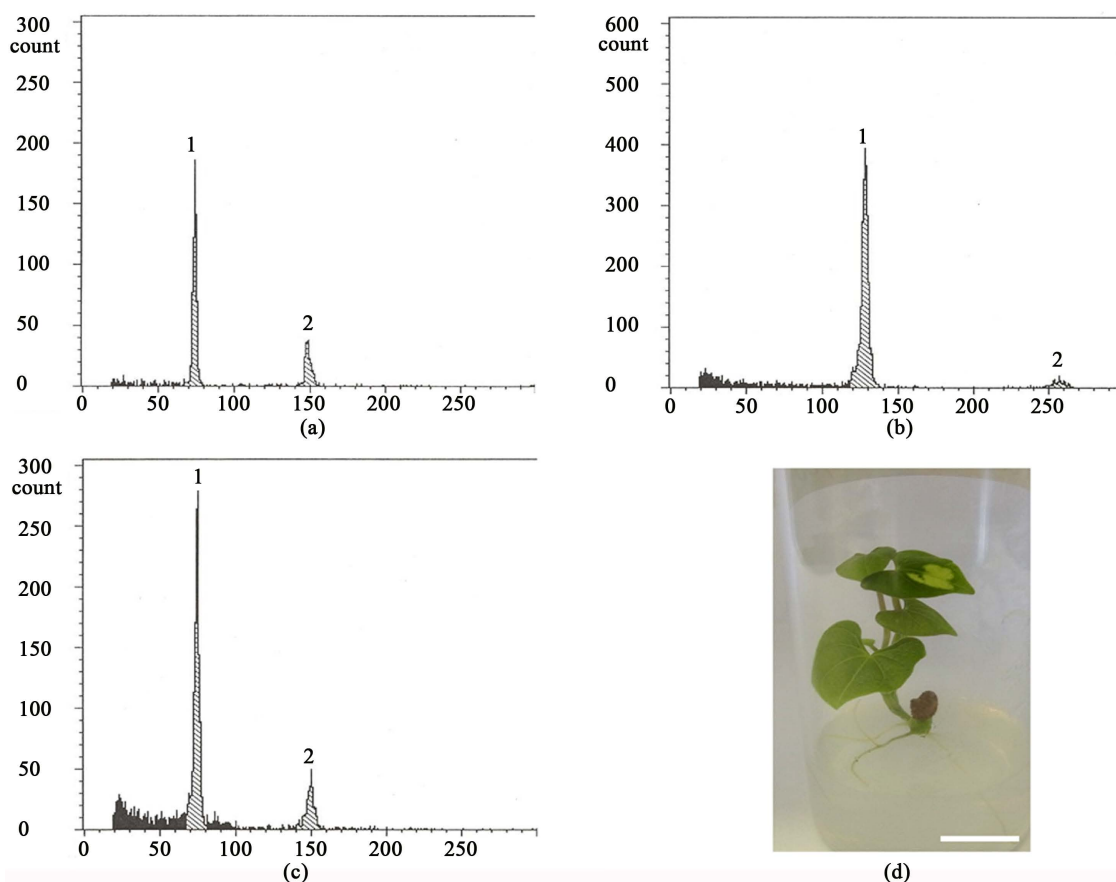


Figure 1. Flow cytometric profiles of (a) *D. rotundata*; (b) *D. bulbifera*, and (c) plantlet between *D. rotundata* × *D. bulbifera*; (d) Obtained plantlet derived from ovule from *D. rotundata* × *D. bulbifera*.

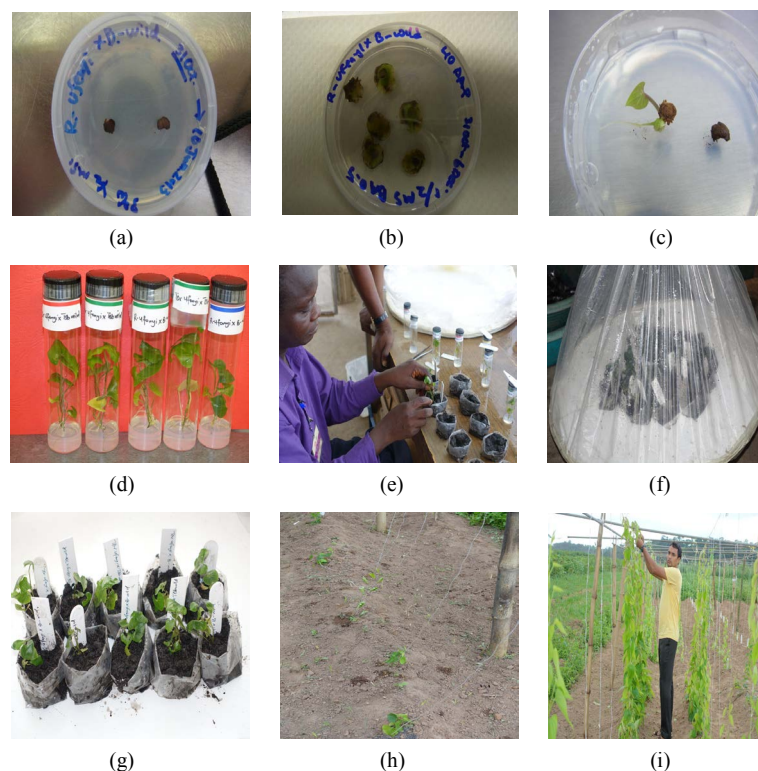


Figure 2. *In vitro* regeneration of *Dioscorea rotundata* × *Dioscorea bulbifera* through ovule culture. *In vitro* cultured ovule on 1/2 MS + 3% sucrose after 3 weeks (a) (b); Germinated ovule from 7 months cultured ovule (c); *In vitro* multiplied shoots and roots of *D. rotundata* ufenyi × *D. bulbifera* wild (d); Acclimatized *in vitro* plantlets and hardening of interspecific crosses of *Dioscorea rotundata* × *Dioscorea bulbifera* (e) (f); Hardened *in vitro* plantlets (g); Regenerated interspecific *in vitro* plantlets of *Dioscorea rotundata* × *Dioscorea bulbifera* in the field (h) (i).

Interspecific crossing is the viable method and makes it possible to obtain *Dioscorea* hybrid plants within *D. rotundata* ufenyi × *D. bulbifera* wild through ovule culture. This regenerated cross is strategically important for the yam breeding of other *Dioscorea* species to increase the genetic gain and gene pool for this crop and also important as a tool of rapid propagation and accelerated breeding in yams.

4. Conclusion

Germination was observed from seven-month cultured ovules which was 40 days after pollination (DAP) of a cross between *D. rotundata* ufenyi × *D. bulbifera* wild (Figure 1(d)). Flow cytometric analysis was used to identify hybridity. The fluorescence intensity of the obtained progeny was closely related to one of the parents' fluorescence (Figures 1(a)-(c)). This indicated that the observed progeny may be an apomictic tissue from an ovule parent of *D. rotundata* ufenyi.

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