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# *In Vitro* Multiplication Rate and Pot Yield of Cryopreservation Derived Buds of Yams (*Dioscorea* Spp)

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## Abstract

One of the principal objectives of the germplasm preservation is to maintain their unique genes combinations. A study was conducted in the laboratory and glass house to evaluate the multiplication rate of *in vitro* cultured plantlets, several morphological and yield characters of glass house pots grown plants derived from cryopreservation using modified droplet method on yams (*Dioscorea* spp.). In vitro MR was similar among *in vitro* culture, PVS2 treatment and cryopreservation derived plantlets for *D. polystachya* and *D. cayenensis*. *In vitro* only derived plantlets exhibited significantly lower MR in comparison to the two other treatment variants for *D. bulbifera*. This was, however, could be attributed to physiological effects which will be recovered in further sub-culture cycles. The morphological characters, number of tuber per plant and tuber' harvest weight per plant were constant for plant derived after cryopreservation and *in vitro* culture (control) for *D. cayenensis*. Modified droplet method is, therefore, reliable for the cryopreservation of yams germplasm and maybe other crops.

**Keywords:** Dioscorea; Cryopreservation derived; *In vitro* multiplication rate; Pot yield

## Introduction

*In vitro* maintenance has been one of the methods implemented in the conservation of yams [1-5]. This method reduces the risk of germplasm losses due to operational error, pest and diseases, climatic changes, as well as the demand for ground area and labour intensive, beard by the field maintenance which is traditionally implemented in the conservation of seedless, recalcitrant and semi-recalcitrant seeds plants [6-10].

There is, however, some discussion, that alteration in the genetic constitution of the conserved materials might occur through the *in vitro* maintenance. Genetic variability generated during tissue culture (somaclonal variation-[11]) is well documented [12]). Imposition of environmental stress or minimal growth during the *in vitro* maintenance as well as type of plant materials used and regeneration modes such as callus and protoplast are some of the frequent factors which could lead to the genetic changes [12 ,13]). Harding [14,15] found that potato shoot tips grown in mannitol-supplemented medium, indicated a DNA hypermethylation, which could be attributable to an adaptive response to condition of high osmotic stress. Since the methylation status may be inherited and alter the phenotypes of the subsequent progenies, this finding has a significant implication to the use of tissue culture techniques in the genetic conservation of potato germplasm [15].

Assuming that all physiological processes are in a 'stand still' stage at cryogenic temperature (-196 for LN -[16]), the materials cryopreserved should retain their genetic constitution upon recovery. This assumption, however, has to be empirically verified since cryopreservation involves

many steps including tissue culture as a transit phase, preconditioning, cryoprotection, cooling and rewarming, recovery and plant regeneration [14] each of which has its own effect on the genetic stability even with the use of explants reportedly stable such as meristem shoot tips [12]. For instance, dimethylsulfoxide (DMSO) in 2 to 10% solution was predicted to be involved in generating a variety of genetic and/or epigenetic changes [17].

Assessment of genetic stability has been performed at the genetic level covering chromosomes and restriction enzymes generated DNA fragment analysis as well as at the phenotypic level including protein electrophoresis, secondary products and morphological (quantitative and qualitative) characters [18,19]). Studies regarding genetic stability of cryopreseration derived materials have been implemented on a number of plant species including some medicinal plants [20], strawberry and raspberry [21,22]), citrus [23]), sugarcane [24], kiwi and grape [25], banana [26]) and yams species of *D. deltoidea* [27], *D. bulbifera* [28]) and *D. floribunda* [29]. The most intensive study, however, has mainly involved potato (*Solanum tuberosum* L.) germplasm where various techniques have been implemented [14,15,19,30-34]. A wide and in-depth discussion regarding genetic stability after cryopreservation is provided by Harding [35].

Three of four species of *Dioscorea* spp. used in our previous study in refining the protocols for yams species using modified-droplet method were successfully recovered into plantlets and transplanted to the glass house. The present report reveals the results of the experiments comparing the *in vitro* multiplication rate (MR) of cryopreserved (+LN), cryoprotectant treated (-LN) and plantlets derived from *in vitro* culture only as well as several phenotypic characters including morphological and yield characters, observed on the glasshouse-grown plants derived from *in vitro* culture and cryopreserved apical buds.

## Materials and Methods

### Plant Materials

Study on the *in vitro* MR involved three genotypes of yams representing three species, namely Yam 16 (*D. bulbifera* L.), Yam 21 (*D. polystachya* Turcz.) and Yam 58 (*D. cayenensis* Lam.) whilst the study on morphological and yield characters was done only on Yam 58 (*D. cayenensis*). The history, the *in vitro* establishment and maintenance of the materials were described in detail in the previous reports [36-38].

### Cryopreservation Procedure

Six weeks old *in vitro* plantlets cultivated in MS [39]) medium supplemented with 2.0 mg/l 6-benzylaminopurine (BAP), 0.1 mg/l α-naphtaleneacetic acid (NAA) designated as M2 medium plus 3% (w/v) sucrose, 0.2 mg/l activated charcoal (AC) and 1% (w/v) agar were further cold-acclimated for 3 weeks in an alternating thermo-photo-period of 28/5 °C, 12 h. Previously, the materials have been cultivated in the maintenance room at constant temperature of 25-27 °C, 16h/8h light/dark periods and light intensity of 60-80 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by fluorescent lamps. Following cold acclimation, the apical buds (plus first axillary nodes for *D. bulbifera)* were dissected under the light microscope to 2-4 mm in length and used as explants. Subsequent preculture in 15% sucrose was applied for the explants of *D. bulbifera* and *D. polystachya*, and 10% (w/v) for *D. cayenensis*, all in M2 medium with 0.7% (w/v) agar.

After a three-days preculture period, the explants were loaded in solution A (loading solution) for 20 min, followed by in PVS2 for 20 min [40]. The explants were than rapidly cooled in liquid nitrogen after placement in droplets of PVS2 on aluminium foil. Rewarming was done by quickly emerging the explants into MS solution with 3% (w/v) sucrose. The explants were than cultivated in semi-solid medium M2 for one week in dark. Further cultivation was done in semi-solid medium MS without hormones plus 3% sucrose. The same medium was regularly renewed in two-weeks intervals, and the shoot recovery from survived explants was observed up to 4 months. The explants (cooled and control) showing shoot development were transferred into medium M2 + 3% sucrose with 0.2% AC and 1% agar in culture tubes for further multiplication. The detailed protocol is described [38].

In experiments of ten cooled plantlets each, 70% survival followed by 30-50% shoot recovery and subsequent plantlet development were obtained using this protocol for three genotypes [38].

### *In Vitro* Multiplication Rate

Plantlets developed after cryopreservation and cryoprotectant treatment (control) were multiplied to a maximum of 18 plantlets per treatment and genotype, the same number as that of *in vitro* maintained plantlets, for the measurement of *in vitro* MR. The plantlets from these treatment variants (cryopreserved, cryoprotectant treated and the *in vitro* derived plantlets) were maintained in the same maintenance medium and culture condition. MR, defined as number of explants obtained after two months culture divided by number of survived explants, was measured for each of the 18 plantlets. An explant was defined as a single node with one leaf attached obtained from any part of the plantlet excluding the original node [36]. Three consecutive measurements were made for each treatment variant. At the first and the second subcultures, a number



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of plantlets (5-8) were randomly selected and multiplied to maintain 18 plantlets for the next sub-culture.

### Glass House Transplantation and Data Measurement

Cryopreservation and *in vitro* derived plantlets (nine plantlets each) were randomly selected from eight-weeks old micropropagated plantlets and transplanted to the glass house. Upon transferred, the plantlets were treated (by dipping into the solution) with a fungicide mixture of 0.2% v/v Polyram Combi (Active ingredient Metiram; BASF AG, Ludwigshafen) and 0.2% v/v Ridomil (Syngenta Agro GmbH, Maintal) in the ratio of 1:1. In the second week of April, transplanting was performed into small pots (diameter 9 cm) filled with 1:1:1 ratio of compost, peat substrate and sand, respectively. After two months cultivation in the small pots, the plants were transferred to bigger pots (diameter 22 cm) containing the same components as that in the small pots in the ratio of 3:1:1. Watering of the plant was conducted once a day and continued up to the middle of October, when the leaves turned yellow and dry. No staking and/or additional fertilization were given at the first cycle of growth (using in vitro plantlets) at the second cycle of growth (using underground tubers of the first harvest). However, a single staking of rope was used for the twinning of the stem. During the spring and summer (the second week of March to the second week of October), the lowest temperature of the glass house was 20-22°C, and during the fall and winter (October to March), it was maintained at 8-10°C. Harvest was conducted in the middle of March, and the tubers harvested from each pot, were replanted together after data measurements.

Observation on morphological traits was conducted during the growing periods of the plants, and at harvest time for the first and second cycles of growth. Some important traits of stem, leaf and tuber were determined based on the descriptor list for *Dioscorea* IPGRI [41] and compared to the description given by Martin FW [42] and Purseglove JW [43]. Colour description (including colour numbers) of some of the traits was based on the Royal Horticultural Society (RHS) Colour Chart. The number of plantlets, survived upon transplanting, was also indicated as percentage of surviving plantlets. Yield parameters measured were number of tubers and tuber harvest weight per plant. Measurement was taken from all surviving plants.

### Statistical analysis

Data of the experiments on *in vitro* MR and yield of glasshouse-grown plants were subjected to ANOVA since they met the assumption of normal distribution and equal variance. One-Way ANOVA was applied to analyse the *in vitro* MR in each genotype and yield traits followed by the Tukey's test to determine the differences among treatment variants, when the ANOVA indicated significant difference. Statistical analysis was accomplished with the help of the software SigmaStat Version 2.0, SPSS Inc., Chicago.

## Results

### *In Vitro* Multiplication Rate

*In vitro* MR of the cryopreserved, cryoprotectant treated and *in vitro* derived plantlets exhibited significant difference in *D. bulbifera* ($p = 0.002$) but not in *D. polystachya* ($p = 0.546$) and *D. cayenensis* ($p = 0.36$) based on the ANOVA. Tukey's test indicated that MR of cryopreservation and cryoprotectant treatment derived plantlets were significantly higher than that of *in vitro* derived plantlets of *D. bulbifera*. No significant difference of MR was observed between cryopreservation and cryoprotectant treatment derived plantlets in this genotype (Figure 1).

### Morphological and Yield Characters

One hundred percent (100%) of the cryopreservation-derived plantlets survived the transplantation as opposed to 67% of the *in vitro* node culture derived plantlets. Plants derived from the two treatment variations, however, further exhibited normal and similar growth and developments.

Observation on the qualitative traits during the first (from the in vitro plantlets) and the second growing cycles (from tubers) indicated no variation between the two treatment variants. The important traits observed are described as follows (some of the traits can be observed in Fig. 2 and 3): Stem is round in shape, without hairs (glabrous) and twinning anti-clockwise (typical for members of section Enantiophyllum). It is dark green in colour (Green group No.137A and B) and branched at the bottom. Spines are present along the stem but mainly concentrated at its lower part and at the axils of plants developed from tuber. Spines productions, however, were less on the plants developed from the in vitro transplanted plantlets. Leaf is single, broadly ovate in shape with acute apex. Old leaves are dark green in colour (No. 137 A) and the younger ones are yellow green (No. 146 A and B), 7 veins appeared on the lamina. Leaf position is alternate and opposite, and the petiole has wide wings at the bottom. Tuber is cylindrical in shape, has a smooth surface with brown or yellowish skin colour. Tuber sprouting started at harvest time and 78% of cryopreservation derived and 83 % of the in vitro derived plants sprouted after 7 weeks (short dormancy period).



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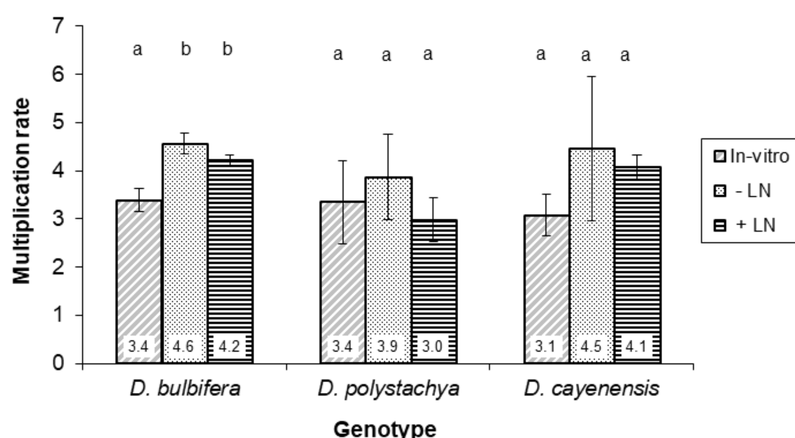


**Figure 1:** *In vitro* multiplication rate of cryopreservation, cryoprotectant (PVS2) treatment and in vitro culture derived yams (*Dioscorea* spp.) of 3 genotypes (species) after measurement of 3 replications with 18 plantlets each. Column on each genotype with the similar letter indicate no significant difference at α = 0.05 based on Tukeys' test. Bars indicate standard error.

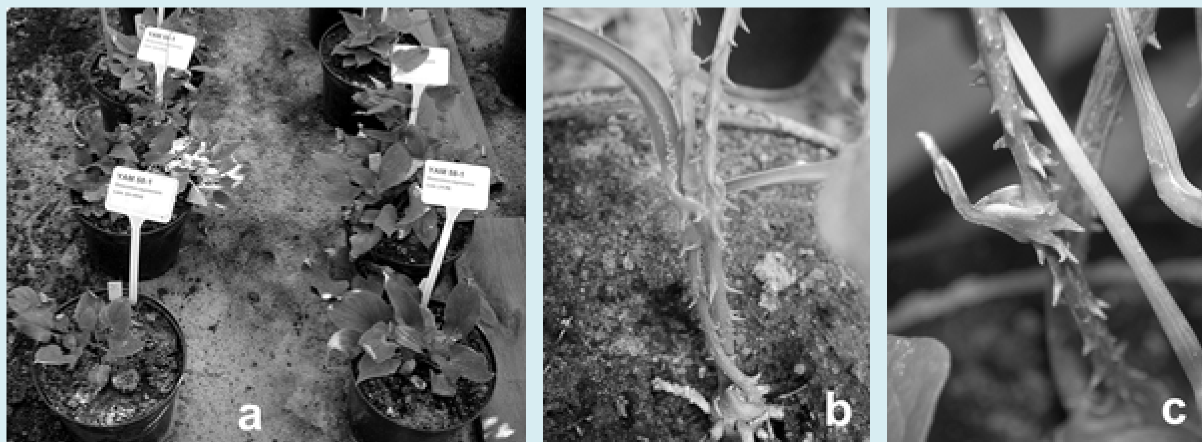


**Figure 2:** Vegetative development of in vitro and cryopreservation derived plants of *Dioscorea cayenensis*, 5-6 months after planting. General condition of plants derived from in vitro culture only (left row) and from cryopreserved apical buds at the first cycle (from in vitro plantlets) of growth (a), and spikes formation on the lower part of the stem at the second cycle (from the harvested tubers) of in vitro only (b) and cryopreservation (c) derived plants.

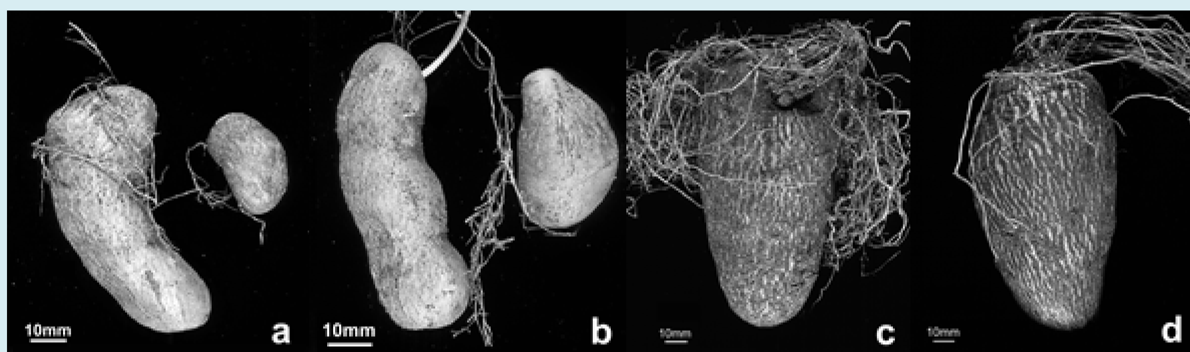


**Figure 3:** Harvested tubers of pot grown *in vitro* node (a and c) cultures and cryopreserved (b and d) apical buds derived plants of *Dioscorea cayenensis* at the first (a and b) and at the second (c and d) cycles of growth.



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A similar number of tubers (average of two tubers per plant) was produced after the two treatment variants. As well, tuber harvest weight exhibited no obvious difference (Table 1) both at the first (46 vs. 44 g per plant) and the second growth cycles (400 vs. 345 g per plant).

| Source of plants | N | | Number of tubers | | Ination of weight (g) | |
|---|---|---|---|---|---|---|
| | C1 | C2 | C1 | C2 | C1 | C2 |
| In vitro | 6 | 5 | 1.8±1.2 | 2.2±1.0 | 45.3±20.8 | 400.4±141.5 |
| Cryopreservation | 9 | 9 | 2.2±1.0 | 2.0±0.9 | 44.9±23.1 | 345.1±164.0 |

N = number of replications. C1 first cycle, C2 second cycle The values are followed by the respective standard deviations.

**Table 1:** Number of tubers and tuber harvest weight per plant of pot-grown *D. cayenensis* derived from *in vitro* node cultures and cryopreserved apical buds at first and second growth cycles.

## Discussion

The effect of cryopreservation using modified droplet (combining droplet and vitrification – 38) method on the physiological and phenotypic characters of yams (*Dioscorea* spp) at the tissue culture and glass house culture stages was studied.

Cryopreservation might have two consequences on the genetic stability of the germplasm; selection effects especially in heterogeneous populations [16] and additional genetic variability due to various steps implemented in the cryopreservation protocol [14,22]. Physiological effects causing phenotypic plasticity may also be the basis of variation in the experimental findings. Physiological effects, however, will decrease in further progeny cycles [19]). Results of this study indicated that none of the mechanisms mentioned above has significantly taken place for cryopreserved materials of two genotypes (*D. polystachya* and *D. cayenensis*) in terms of *in vitro* MR as well as all investigated phenotypic qualitative and yield characters for *D. cayenensis*. Evaluation in further generations might determine whether the alteration in the MR in this study (cases of cryopreservation and PVS2 treatment derived plantlets in *D. bulbifera*) was due to genetic or physiological causes. However, based on our two years study covering ten consecutive subculture frequencies of MR [44], where the fluctuation exists in every cycle of subculture, it may be assumed that the difference in the MR on *D. bulbifera* genetic back ground in this study was of physiological cause and will be recovered in further cycles of subculture.

Investigation on the stability of MR of *in vitro* shoot or node cultures after cryopreservation has not been published for yams. Studies on *Panax ginseng*, however, indicated that roots regenerated from cryopreserved hairy roots, proliferated fewer new lateral roots compared to the control but exhibited no differences in further development [20]. The suppression of proliferation rate was attributed to direct effect arising from cryogenic procedure [20]. Morphological observation of the plantlets regenerated from somatic embryo and further microtuber production on *Dioscorea bulbifera* reported a similarity between cryopreserved and control plantlets [28].

Retainment of plant morphological characters after cryopreservation on the glass house grown plants has been reported for potato [31,32]. Field studies Ahuja S [29] observing several phenotypic characters on *D. floribunda,* reported similar qualitative characters and no significant differences on the quantitative characters between cryopreserved and *in vitro* culture generated plants. Plants regenerated from cryopreserved suspension cells of banana Côte Fx [26] and from cryopreserved calluses of sugarcane Martínez-Montero ME [24]) generally indicated stability in several agronomic and morphological traits. Although there were variations between cryopreserved and control materials on two traits at the first cycle of growth of banana plants, these differences disappeared at the second growth cycle [26]. *In vitro* derived sugarcane plants regardless of their cryopreservation status produced significantly smaller diameter and shorter plant height in comparison to the macro-propagated plants during the first six months of cultivation. These differences disappeared, however, after one year of field growth [24]. In contrast to those studies, a number of quantitative characters including tuber harvest weight, plant height at the flowering stage, length of petiole and breath of terminal leaflet were changed after cryopreservation compared to the tissue culture control plants of potato cv. 'Golden Wonder'[19]. Biometric indicators were also found to be changed after cryopreservation of strawberry, based on the field trials [21].

Except for variation in methylation status (epigenetic change) of ribosomal RNA and nuclear DNA [22] [45], studies on various species confirmed the stability of DNA after cryopreservation. Those studies covered *Solanum tuberosum* shoot-tips cryopreserved using encapsulation-dehydration and droplet methods [32-34], *Atropa belladona* hairy roots and *Papaver somniferum* calli cryopreserved


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using vitrification method [20], *Citrus* spp vitrified calli [23], shoot tips of *Dioscorea floribunda* [29] and embryogenic tissues of *D. bulbifera* implementing encapsulation-dehydration method [28]. Further observations have also indicated stability in both chromosome number and ploidy level for potato [30, 31], *Dioscorea deltoidea* [46] and *Citrus* spp. Hao YJ [23]. At the biochemical level, consistency on the secondary metabolites productions after cryopreservation has been confirmed for several medicinal species such as P*anax ginseng*, *Papaver somniferum* Yoshimatsu K [20] and *Dioscorea deltoidae* [46]. For the species *Atropa belladona*, the secondary metabolite production was fluctuated at the initial culture after cryopreservation but recovered to the same level of untreated roots after further subculture Yoshimatsu K [20].

The practical impact of stability in the MR is that cryopreserved yam germplasm, upon retrieval from cryopreservation, can be directly propagated for the purpose of rapid plantlet multiplication or for germplam exchange with the same number of plantlets as that normally obtained with the *in-vitro* multiplication. Furthermore, in the development of cultural practices of yams' breeding and field production, *in vitro* plantlets of two or more months old, will be transferred to the field or nursery beds in the first year and the tubers produced are selected based on their characteristics and used for the normal production in the next year [47,48]. The similarity in tuber production of plants derived from cryopreserved apical buds and *in vitro* plantlets of *D. cayenensis* in this study indicates that the similar cultural and selection practices for *in vitro* plantlets can be applied directly to plantlets recovered from cryopreservation. The number of tubers per plant produced for *D. cayenensis* in the first cycle of growth (45 g), despite the application of only simple cultural practices (without staking and additional fertilizers), was in the range of that produced in nursery beds (20-250 g) reported for *D. rotundata, D. dumetorum* and *D. bulbifera* [48].

## Conclusions

Cryopreservation is regarded to be the most suitable method for conservation of seedless, semi-recalcitrant and recalcitrant seeds plants. This method, however, involves different steps which could contribute to the alteration of genetic constitution of preserved germplasm especially when the protocol used is not well developed. The present study indicated no alteration in the i*n vitro* MR for two of three genotypes tested, and constant morphological characters, number of tubers per plant and tuber harvest weight per plant of glasshouse pots-grown plants of *D. cayenensis* after cryopreservation. These findings support the concept that cryopreservation, specifically the modified droplet protocol developed for yams, is reliable for the preservation of the genetic constitution of stored yams' germplasm.

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