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Impact of osmotic dehydration on the encapsulated apices survival of two yams (*Dioscorea spp.*) genotypes from Benin

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ABSTRACT

Objective: Cryopreservation is one of the biotechnological methods currently used for long term conservation of plant genetic resources. It requires many steps such as a pretreatment, which involves cells dehydration in order to make them tolerant to desiccation and freezing in nitrogen liquid using high sucrose concentration. The present study aims to establish a protocol for long term conservation of yam germplasm in Benin using encapsulation/dehydration technique.

Methodology and Results: The effects of different sucrose concentrations and immersion durations on encapsulated apices of two yams genotypes (Da93G1 from *Dioscorea alata* and Dcr164 from complex *Dioscorea cayenensis/ D. rotundata*) were tested. The apices were excised from six months old vitroplants on stereoscope and transferred in M1 medium (MS+2g.L^{-1} activated charcoal) prior to encapsulation in alginate (3%) beads with calcium chloride (1.32M). Then, the apices were exposed to osmotic dehydration with two concentrations of sucrose (0.75M and 1.25M) at two durations (24h and 40h) before their culture in M2 medium (MS + 2mg.L^{-1} BAP, 100µg.L^{-1} d'ANA and 2g.L^{-1} activated charcoal).

Conclusion and application: The results showed that 0.75M sucrose increased high survival rate (80%) and high rate of regenerated plant (70%) at 24h of immersion duration. Furthermore, significant difference was observed between the two genotypes response. This work has allowed adopting in further experiments 0.75M sucrose and 24hours of immersion duration in pretreatment of yam apices for the development of cryopreservation techniques for yam conservation in Benin.

Keywords: *Dioscorea spp.*, sucrose, osmotic dehydration, regeneration, immersion duration, cryopreservation, Benin.

INTRODUCTION

Yam belongs to the genus *Dioscorea* (Dioscoreaceae). The genus contains about 600 species with more than 10 species cultivated for food and for pharmaceutical purposes (Coursey, 1976; Aké Assi, 1998). Six species of them are more important: white yam (*Dioscorea rotundata*), water yam (*Dioscorea alata*), yellow yam (*Dioscorea cayenensis*), trifoliate or bitter yam (*Dioscorea dumetorum*), aerial yam (*Dioscorea bulbifera*) and Chinese yam (*Dioscorea esculenta*) (Ng and Ng 1994). In West Africa, and particularly in Benin, yam is one of the staple food crops contributing to food

security and poverty reduction. In addition to its economic and nutritional values, there is a preeminent socio-cultural dimension of the use of yam across most of the sociolinguistic groups in Benin (Dansi *et al*. 2000, Zannou *et al*. 2009).

In the whole farming system, the conservation of yam genetic resources remains the main challenge to take up. Indeed, traditionally, yam germplasm are maintained in field genebanks as living collections because genotypes are either highly sterile, produce heterogeneous seeds, or possess tubers which have poor storability (Zoundjihekpon 1993, Mandal *et al*. 1996). Since yam is predominantly vegetatively propagated, conservation of its germplasm using conventional method of field maintenance poses various problems. Field maintenance involves high costs and important post-harvest lost due to pest/pathogen attacks and natural disasters (Mandal *et al*. 2007, Houedjissin *et al*. 2010). Without an efficient technique of conservation, there is an important risk of genetic erosion of yam genetic resources. It is important to find out an alternative way for best storability of the yam genotypes. *In vitro* plant tissue and organ culture can be an alternative way to solve this problem (Ahanhanzo *et al*. 2010). However, the maintenance of large *in vitro* collections is time consuming and the risks of contamination and somaclonal variation increase with time (Podwyszyńska, 2005). Cryopreservation techniques presently offer the most promising approach for long term conservation of germplasm under conditions favouring high genetic stability and minimal maintenance requirements (Mandal *et al*. 1996, Engelmann *et al*. 2000). Various cryopreservation techniques have been developed and protocols have been established for several plant species such as *Yam* (Mandal *et al*. 1996, 2007), *Elaeis guineensis* (Engelmann, 1995), *Solenostemon rotundifolius* (Niino *et al*. 2000), *Pelargonium* (Dumet, 2002, Gallard, 2008), *Musa* (Panis, 2009), *Manihot esculenta* Crantz (Dumet, 2011). According to the available literature, two techniques are reported to be successfully for cryopreservation (droplet vitrification and encapsulation/dehydration).

Encapsulation/dehydration method is critically dependent upon the optimization of dehydration and meristems desiccation tolerance is an important survival factor (Barbara *et al*. 2001). On top of that, the transfer of cryopreservation protocols from one laboratory to another may result in variable responses, reflecting differences of physiological status of the cultures in response to desiccation or culture conditions (Barbara *et al*. 2001).Thus far, in Benin, cryopreservation has not yet been applied in research programs. The aim of this study was to establish a protocol for long term conservation of yam germplasm in Benin using encapsulation/dehydration technique. To this end, the effects of different sucrose concentrations and immersion durations on encapsulated apices of two yams genotypes were tested.

MATERIEL AND METHODS

Biological material: Two yams genotypes (accession Dcr164 belonging to the complex *Dioscorea cayenensis/D. rotundata* and accession Da93G1 belonging to the species *Dioscorea alata*) were used in this study. These genotypes were collected at the *in vitro* genebank of the Laboratory of Genetic and Biotechnology of the Faculty of Sciences and Techniques (University of Abomey-Calavi, Republic of Benin). The choice of these two accessions is guided by the fact that their tubers are more sensible to pest/pathogen attacks. In addition, they are well adapted to microcutting response.

Methods

Preparation of microshoots for encapsulation: Microshoots were obtained from six months old vitroplants and cultivated two weeks in Petri dishes containing semi-solid Murashige and Skoog (1962) medium (MS) supplemented with 2g.L^{-1} activated charcoal. Each Petri dish contained about 45 at 50 microshoots.

Apices excision: The apices were excised from auxiliary buds of microshoots after two weeks culture in sterile condition over laminar flow. The excision was made on stereoscope using sterile scalpel blade N°11. Then, the apices were laid on semi-solid hormone-free MS medium supplemented with 100g sucrose, 7g bacteriological agar and 2 g.L^{-1} activated charcoal (M1 medium) and were left one day in the dark at 25°C.

Encapsulation of apices and immersion in sucrose solution: The apices were moved in alginate sodium (3%) and were dropped in polymerization solution

(calcium chloride 1.32M) in order to develop the beads, which contained one apex each. The beads were dry out on sterile filter paper before their immersion in high sucrose solution (0.75 M and 1.25 M) during 24 hours and 40 hours over agitation (100rpm). After exposing on high sucrose, the apices were grow in Petri dishes on semi-solid MS medium supplemented with 2mg.L^{-1} BAP, 100µg.L^{-1} NAA and 2g.L^{-1} activated charcoal (M2 medium). Each dish contains 10 encapsulated apices as showed in Table1. All media were sterilized for 15min at 115°C and the pH was adjusted to 5.7 before sterilization. The dishes with encapsulated apices were maintained in dark for one week then transferred to fluorescent light (5.000 µmol.m^{-2}.S^{-1}) for 16h per day at 27 ± 1°C. The apices were cultured for 8 weeks and the following parameters were evaluated:

- The survival rate was assessed three weeks after culture in M2 medium by counting the number of growing apices. An apex was considered alive when it was green.
- The necrosis rate was determined three weeks after culture in M2 medium by counting the number of necrosis apices which appeared totally brown, not hairy and showed no sign of regrowth.
- The regenerated rate was expressed by a percentage of a total of growing apices forming shoots and roots within 8 weeks.
- The callus rate was determined three weeks after culture in M2 medium by counting the number of apices producing callus on the scar zone.

Table1. Treatments of the encapsulated apices of the two tested yam accessions

Treatments (Concentration of sucrose + immersion duration)	**Number of encapsulated apices per Accession**	
	Da93G1	**Dcr164**
T1 **:** 0.75M + 24h	10	10
T2 : 1.25M +24h	10	10
T3 : 0.75M + 40h	10	10
T4 : 1.25M + 40h	10	10

Experimental design: To test the effect of the different treatments, treatments were plotted in a completely randomized design with two replicates. Each treatment included 20 apices (10 apices per accession). The data were analyzed using SAS program version 9.0 and if ANOVA indicated significant ($P<0.05$) differences, the means (expressed as percentage) were separated using Student, Newman and Keuls' test.

RESULTS

Response of encapsulated apices: The Figure 1 illustrates the response of encapsulated apices to different treatments. After two to eight weeks of culture, four various reactions were noticed:

- Browning apices indicating that the apices were immediately dead by the tissues necrosis,
- Swelling apices which were still green indicating activated cells multiplication,
- Callus production,
- and cells differentiation to produce shoots.

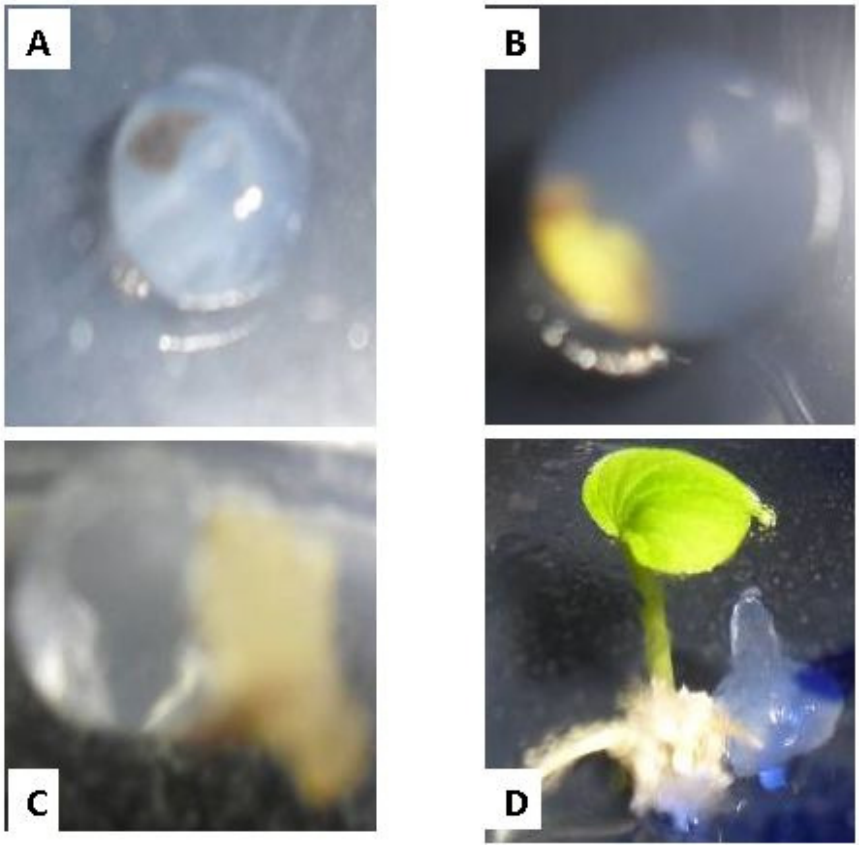

Figure 1: Reaction of encapsulated apices after sucrose (0.75M) treatment (from accession Da93G1)
A- Necrosis apex ; **B**- swelling apex ; **C**- Callus apex ; **D**- Regenerated apex

Effect of sucrose concentration and immersion duration on apices survival: Most of the encapsulated apices treated from 0.75M sucrose survived (treatments 1 and 3) within 2 weeks independently to immersion duration (Figure1a). The high level of survival (90%) was obtained from the accession Dcr164, but was not significantly different compared to accession Da93G1 (70%). In contrast, most of the apices which were exposed in 1.25M sucrose dead (treatments 2 and 4). Besides, the long immersion duration increased the death rate of apices (Figure1b). When the apices were exposed during 40 hours in 1.25M (T4), the high survival rate was 20% with both of accessions while this rate ranged from 20% (Dcr164) to 50% (Da93G1) with 24 hours in the same sucrose concentration (T2) with significant difference between accessions. On the other hand, the media containing high sucrose (1.25M) increase mostly necrosis apices (treatments T1 and T2) compared to those media that have less sucrose (0.75M) according to treatments T1 and T3 (Figure1b). Furthermore, maximal necrosis apices rate (80%) was unregistered in treatment T4

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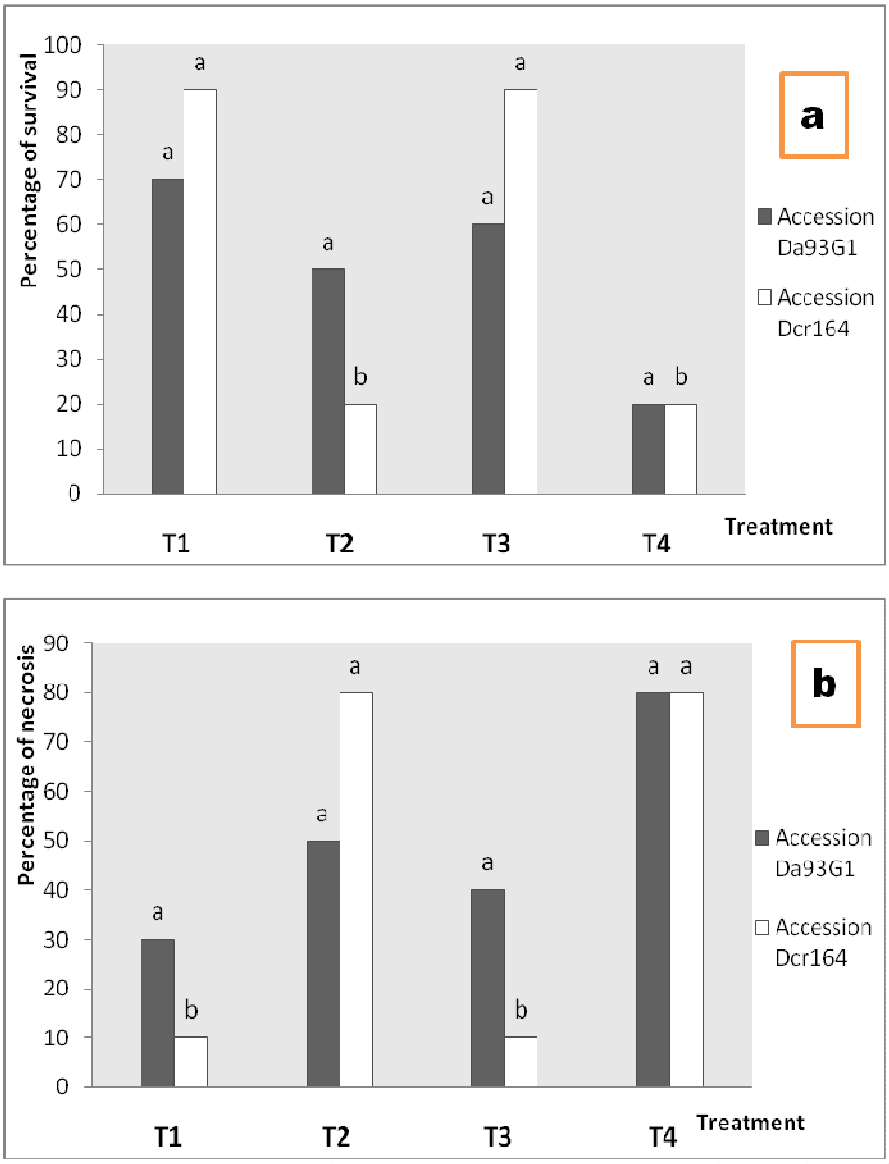

Percentages in the same color followed by the same letters do not differ significantly (p< 0.05).

Figure 2: Survival (a) and necrosis (b) rates of yam apices in the different treatments.

Effect of sucrose concentration and immersion duration on callus induction: Callus was observed within two weeks after apices culture in M2 medium. Irrespective of treatments, none of the apices of the accession Da93G1 had callus (Table 2). However, callus proliferation was noticed on apices belonging to accession Dcr164, which show the highest percentage of callus. Furthermore, a high sucrose concentration increased callus formation (80% with 1.25M sucrose against 20% with 0.75M). Significant difference ($P<0.001$) was observed with the effect of immersion duration on callus induction. Proportion of callus ranged from 0% to 20% respectively after a 24 hours and 40 hours immersion in 0.75M sucrose. Similar observations were noticed with 1.25M sucrose. Overall, an average of callus (80%) was observed after 40 hours against 50% after 24 hours.

Table 2: Effect of different treatments on callus formation of the two tested yam accessions

Treatments (Concentration of sucrose + immersion duration)	**Callus rate (%)**	
	Da93G1	**Dcr164**
T1 **:** 0.75M + 24h	0^{a}	0^{c}
T2 : 1.25M +24h	0^{a}	50^{ab}
T3 : 0.75M + 40h	0^{a}	20^{bc}
T4 : 1.25M + 40h	0^{a}	80^{a}

Percentages in the same row followed by the same letters do not differ significantly (p< 0.05).

Effect of sucrose concentration and immersion duration on regeneration: The Table 3 shows the regeneration of apices on different treatments within 8 weeks culture. The results indicate that the accession Da93G1 (*Dioscorea alata*) has the highest percentage of regeneration (70%) with 0.75M sucrose treated for 24hours (treatment 1) while the 1.25M sucrose treatment induced only slightly regeneration of apices (10%) at equal immersion during (treatment 2). None of the apices belonging to accession Dcr164 was regenerated when the sucrose concentration is important (1.25M).On the other hand, results indicated that, a long duration exposition decreased the regeneration of apices. In fact, with 1.25M sucrose, 10% of apices belonging to accession Da93G1 were regenerated after 24hours immersion (T2) while none of them was regenerated after 48 hours exposition (T4). The Figure 3 shows the pictures of regenerated apices and non-regenerated apices.

Table 3: Effect of different treatments on regeneration of the two tested yam accessions

Treatments (Concentration of sucrose + immersion duration)	**Regeneration level (%)**	
	Da93G1	**Dcr164**
T1 **:** 0.75M + 24h	70^{a}	20^{a}
T2 : 1.25M +24h	10^{b}	0^{a}
T3 : 0.75M + 40h	20^{b}	10^{a}
T4 : 1.25M + 40h	0^{b}	0^{a}

Percentages in the same row followed by the same letters do not differ significantly (p< 0.05).

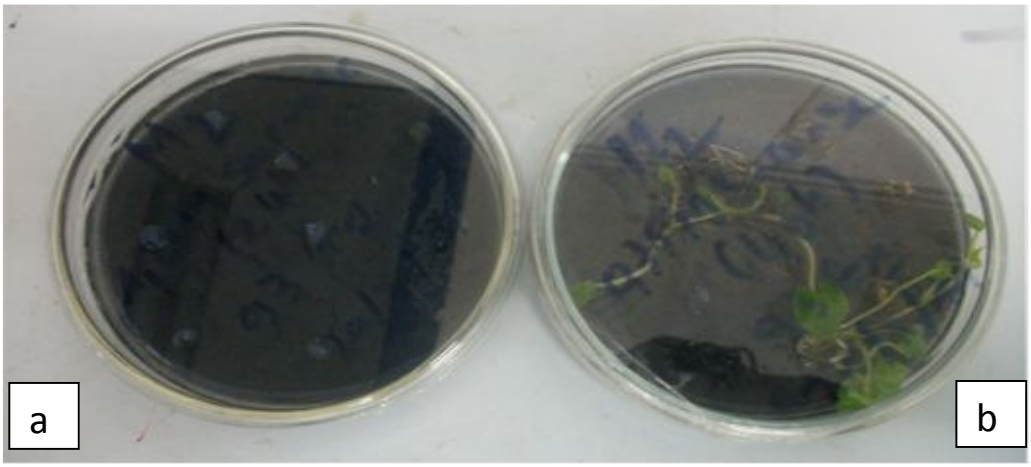

Figure 3: Apices response belonging to accession Da93G1
(a) = non-regenerated apices, b= regenerated apices.

DISCUSSION

Several studies showed that the level of water in alginate beads is an important factor for survival of encapsulated apices. Consequently, an important water in beads induce lethal ices after freezing in liquid nitrogen who

damage cells while low water induce high dehydration and subsequently the cells death (Baudot *et al*, 1998).The results obtained in this experiment showed that the accession Dcr164 (*Dioscorea cayenensis)* recorded the highest rate of survival (90%) with 0.75M sucrose while Da93G1 accession (*Dioscorea alata*) presented 70%. Despite the fact that no statistical difference was observed between them, the survival rates of apices varied with sucrose concentration and the genotype (treatment 2). These results are congruent with previous reports on *Pelargonium* species which illustrated genotypic differences (survival and regeneration rates) in response to cryopreservation protocols Gallard (2008). Dumet *et al* (2011) also showed that *Manihot* meristems surviving cryopreservation depends on accessions.

With regards to sucrose molarity, 0.75M sucrose increased high level survival comparing to 1.25M sucrose. This result supports Dumet *et al* (2002) who reported that 0.75M sucrose molarity optimizing tolerance to desiccation and cryopreservation of numerous plant tissues. The high rate of apices death could be explained by the excessive osmotic dehydration induces by high concentration of the sucrose which causes cells plasmolyze (Finkle *et al*. 1985). According to Stranzel *et al* (1988), the process of sucrose accumulation depends on its concentration in the medium: at concentrations lower than 10^{-3}M, sucrose was hydrolyzed before absorption whereas at higher concentrations, sucrose uptake proceeded through hydrophilic membrane domains. They further noticed that sucrose appears to have a very specific action for the acquisition of tolerance to freezing in liquid nitrogen with tissues at intermediate water contents. Intracellular concentration of sucrose may be sufficient to protect cell structures, either by allowing vitrification of intracellular solutes at positive temperature (Williams and Leopold, 1989) or by stabilizing membranes and proteins (Crowe and Crowe, 1986).

Based on the present results, callus induction depends in large part on genotype and less evidently on high sucrose concentration. However, the mechanism of callus induction is still poorly understood. The regeneration capacity of both accessions tested varied with different treatments. Similar results were reported on cryopreservation of *Pelargonium* cultivars indicating that only cultivar 'Balcon Lilas' developed best aptitude to regenerate after encapsulation/dehydration in contrast to others cultivars which apices induced callus with hyperhydric shoots (Gallard, 2008). Ahouanse (1996) also showed that the number of germinated embryos of *Phaseolus Vulgaris L*. decreased with high sucrose concentration in the media. In contrast, Engelmann (1995) noticed that an additional pregrowth treatment of embryos on a medium with high sugar concentration may also prove beneficial to increase the resistance of embryos to desiccation and freezing. Therefore, the response of the increase of osmotic pressure may be species specific.

CONCLUSION

The present study defined the optimal conditions for the pretreatment of yam apices of two Beninese genotypes prior to the freezing in nitrogen liquid. Before their immersion in nitrogen liquid, at ultra low temperature, cells need to be devoid of intracellular liquid in order to avoid lethal ices formation, which is prejudicial to their survival and regeneration. This work permits to adopt in the further experiment 0.75M sucrose and 24hours of immersion duration in pretreatment of yam apices for the development of cryopreservation techniques for yam conservation.

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