Research Paper

Germinability of *In-Vitro* Conserved *Dioscorea* Microtubers in Different Storage Conditions

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Abstract

***Dioscorea* is a traditional tuber crop species. Sri Lanka has eleven local and one introduced *Dioscorea* species. Two hundred and five *Dioscorea* accessions have been conserved in *in-vitro* and *in-vivo* conditions at the Plant Genetic Resources Center (PGRC), Sri Lanka as a national mandatory duty. *In-vitro* conservation of *Dioscorea* needs frequent sub culturing hence conservation cost is very high. Conservation of *Dioscorea* as microtubers is a cost effective alternative method. Therefore, this research was conducted to identify the optimum microtuber size and storage temperature to develop a method for microtuber conservation. Four *D. alata* accessions; *Raja ala*, *Angili ala*, *Kiriwel ala*, *Peni ala* and one *D. bulbifera* accession; *Bakamunu ala* were selected for this research. *In-vitro* initiated microtubers having four different microtuber diameter categories of five selected accessions were tested under three storage temperature levels. The experiment was laid-out as a completely randomized design with five replicates. After one year of storage period germination was induced and germinability was recorded. ANOVA was performed to analyze the data. At 25 °C storage condition microtuber categories of diameter > 5 mm of all selected accessions couldn't be conserved for more than 4.5 months. The microtubers with the diameter < 5 mm at any tested temperatures had zero germination. All selected accessions having microtuber diameter >5 mm can be conserved at 1 °C or 4 °C temperatures for one year with a very low germination (3.68 %). Further studies have to be conducted to improve the germinability of conserved microtubers of *Dioscorea* spp.**

Keywords: ***Dioscorea*, Dormancy, *In-vitro* storage, Microtubers, Tuber crops.**

Introduction

Dioscorea or yam (*Dioscorea* spp.) is an important multi-species tuber crop in Sri Lanka. It is a polyploids with varying chromosome number 2n= 4x = 80 (Sartie and Robert, 2011). Sri Lankan *Dioscorea* germplasm consists of eleven indigenous and one introduced species (Jayasuriya, 1995). The most popular *Dioscorea* spp. is *D. alata* or *Mahawelala* whereas *sculenta* is also a prominent species. *Dioscorea rotundata* is a species introduced from Nigeria. *Dioscorea pentaphylla* is a wild species recorded as rarely cultivated in Sri Lanka and home garden level. *Dioscorea koyamae jayasuriya* and *D. trimenii* are endemic to Sri Lanka while *D. spicata* is a species which is vulnerable to extinct (MOE, 2012). *Dioscorea bulbifera, D. tomentosa*, and *D. oppositifolia* also present in Sri Lanka as low concern species (MOE, 2012).

Dioscorea tubers contain important dietary, medicinal and herbal properties (Gunasena, 1989). As a seasonal crop, it can be grown in *Yala* season and harvest in *Maha* season. One of the special characters of *Dioscorea* tuber is, it remains in dormancy before sprouting. The interest to cultivate *Dioscorea* spp. enhances due to its easy management, minimum pest and disease attack, medicinal value and suitability in marginal lands thus increase the demand (DOA, 2006).

A rich diversity among *Dioscorea* collections stipulates the need for conservation to prevent genetic erosion and to enhance the use of these genetic resources for utilization. (Ng and Ng, 1994; Acheampong, 1996). The two basic approaches for plant germplasm conservation are; *in-situ* and *ex-situ*. *In-situ* conservation aim s at conserving germplasm in its natural original habitat where as *ex-situ* conservation is the conservation of components of biological diversity outside their natural habitats. Field conservation is one of the *ex-situ* methods that is required considerable space, maintenance and time however, the plants can be lost due to pest and disease, poor sprouting, unsatisfactory storage of tubers, drought and poor handling (Okoli, 1991; Acheampong, 1996). *In-vitro* methods of plantlet storage at reduced temperature to slow down their growth rates, thereby extending the viable storage period, is also used for several species of *Dioscorea*.

Dioscorea accessions are conserved *in-vitro* in the Plant Genetic Resources Center (PGRC) as a national conservation mandate. Up to 2021, PGRC has been conserved 205 accessions in *in-vitro* form as shoot tips and *in-vivo* form in pots. These accessions represent most of the Sri Lankan *Dioscorea* germplasm including wild relatives, landraces and cultivated spp. Sub-culture period of *in-vitro* conserved *Dioscorea* cultures is very short ranging from 4 - 6 months. Hence frequent sub-culturing is needed. It is laborious, costly and prone to human errors such as mislabeling. Therefore, it is essential to explore alternative methods to reduce the operational cost.

Naturally forming organs such as microtubers of some crops can be conserved *in-vitro* with a long passage period (Morufat, 2009; Mandal *et al*. 2008) more than one year. Microtubers are small tubers originating from the *in-vitro* grown plant tissues that offer several advantages over *in-vitro* grown plants. They are more tolerant to light and temperature

variations than *in-vitro* plantlets and do not need frequent sub-culturing. In addition microtubers are easy to handle over *in-vitro* plantlets (Mantell and Hugo, 1989). They can be rooted directly and transferred to the soil mixtures. Therefore, microtubers are a better alternative than *in-vitro* axillary bud culture which requires hardening and acclimatization. Hence, the study was performed to identify the optimal storage temperature, storage period and microtuber size of selected five *Dioscorea* accessions with aiming long term conservation of *Dioscorea* germplasm using microtubers as an *ex-situ* conservation tool.

Materials and Methods

This research was conducted at the *in-vitro* conservation laboratory of the PGRC, Gannoruwa from August 2017 to December 2019.

Explants

Among the 205 *Dioscorea* accessions conserved at the PGRC, commonly available four *D. alata* accessions; *Raja ala* (Accession No. BTD 123), *Angili ala* (Accession No. BTD 29), *Kiriwelala* (Accession no. BTD 11), *Peni ala* (Accession no. BTD 22) and a wild variety *Bakamunu ala* (Accession no. BTD 117) from *D. bulbifera* species were selected for this research.

Dioscorea tuberization

Healthy, disease free axillary buds (explants) were initiated in MS medium (Murashige and Skoog, 1962) supplemented with 2 mg L^{-1} Indole 3 Acetic Acid (IAA), 1.5 mg L^{-1} Kinetin and 3 % sucrose. Initiated *Dioscorea* shoots were multiplied in MS medium fortified with 0.5 mg L^{-1} α-Naphthalene Acetic Acid (NAA), 2 mg L^{-1} 6-benzylaminopurine (BAP) and 3 % sucrose (Edirisinghe *et al*. 2017). The *in-vitro* multiplied shoots from selected accessions were cultured aseptically in half-strength MS medium supplemented with 1 mg L^{-1} BAP, 0.5 mg L^{-1} NAA, 0.1 mg L^{-1} gibberelic acid (GA_3) and 80 g L^{-1} sucrose aseptically under laminar flow cabinet. Treated samples were kept in the incubation room until they gave rise to microtubers. All the cultures were maintained at 24 ± 1 °C temperature and 60 to 70 % relative humidity under 16 hours light / 8 hours dark photoperiod with the light intensity ranging from 37 to 46 µ mol m^{-2} s^{-1}. pH of all the media were adjusted between 5.7 to 5.8 and 8 g L^{-1} of agar powder was added to solidify the culture initiation medium. Multiplication and tuberization media were solidified by adding 11 mg L^{-1} of agar. Seven milliliters of culture initiation medium was poured into 25 mL culture vials. Thirty milliliters of multiplication medium was poured into each 150 mL small jam bottles while 40 mL of tuberization media were added into each 350 mL large jam bottles.

Microtuber harvesting

After tuber formation, cultures were incubated under same culture conditions until tubers become mature or brown in color. Then tubers were harvested aseptically separating them from the plant under the laminar flow cabinet. The diameter of each tuber was measured using a grain meter. All the harvested microtubers were grouped according to the accession number. After that all those microtubers were dried under the laminar flow for eight hours per day for 5 days. In each day, after completion of eight hours, the dried microtubers containing petri dishes were closed and kept a side in the laminar flow.

Testing of microtuber storage period

Microtubers from each accession were further sorted into four different size categories according to the diameter as; < 3 mm, 3-5 mm, 5-7 mm and > 7 mm. Then laminar flow dried microtubers were placed in sterilized test tubes (12.5 cm height, 1.5 cm diameter) and labeled. These microtubers were stored under three different temperature levels; 1 °C (dark condition), 4 °C (100 Lux) and 25 °C (2500 Lux). A set of sample was stored in base collection chambers of seed genebank, PGRC to provide 1 °C temperature. 4 °C and 25 °C storage temperatures were provided by keeping the samples in the bottle cooler (Sanyo SSR 500) and *in-vitro* culture incubation unit respectively. After storing, microtuber sprouting was observed in intervals 4.5 months and 12 months.

Germination of conserved microtubers

After completion of 4.5 months and 12 months, conserved microtubers were taken out from the relevant temperatures and kept under room temperature for one week. Then microtubers were transferred into 350 mL jam bottles containing sterilized distilled water soaked tissues. The bottles were covered with polyprophylene polythene and further sealed with para-filim and placed in an incubator at room temperature until tuber germination occurs. When a shoot reached 1 cm length, those microtubers were considered as germinated tubers.

Data analysis

All the *in-vitro* experiments were planned as Completely Randomized Design (CRD) with factorial experiments. Each treatment consisted of five replicates and seven microtubers per replicate. The collected data were analyzed by Analysis of Variance (ANOVA) procedure using the Statistical Analysis Software (SAS) package (Portable SAS version 9) and means were separated by Duncan's Multiple Range Test (DMRT).

Results and Discussion

After four and half months, microtubers stored at 25 °C exhibited *in-vitro* germination in stored test tubes itself. Therefore tuber germination percentage of microtubers maintained at 25 °C was tested in four and half month period. Significant differences were observed between the accession no. 117 *Bakamunu ala* and accession no. 11 *Kiriwelala* (Table 1). Other accessions did not show differences in mean tuber germination after four and half month conservation period at 25 °C under *in-vitro* conditions.

According to the Table 2, mean germination percentage of tuber categories 5-7 mm and > 7 mm diameter showed significant difference in all selected accessions at 25 °C temperature. But none of the tested microtuber under categories 3-5 mm and <3 mm diameter show germination even at six months period (Observation).

Table 1. Percentage of mean germination of microtubers from different accessions stored at 25 °C after four and half month.

Accession no.	Mean microtuber germination %
117	33.5[a]
29	30.5[ab]
123	30.0[ab]
22	29.0[ab]
11	28.0[b]

Mean values followed by the same letter are not significantly different at 0.05 probability level.

Table 2. Percentage of mean germination of deferent microtuber categories stored at 25 °C after four and half months

Accession no.	Mean microtuber germination %
> 7	69.6[a]
7-5	51.2[b]
5-3	0
< 3	0

Mean values followed by the same letter are not significantly different at 0.05 probability level.

Ovono et al. (2010) report that germination of *D. rodundata* microtubers (2.5 cm length) stored at 25 °C resulted 35 % sprouting after 2 weeks and 100 % germination occurred at 8 weeks. Gopal *et al*. (2004) had reported contradictory findings with potato microtubers. They had concluded that microtubers stored under diffused light condition; 20 µ mol m^{-2} s^{-1} light intensity with cool white fluorescent lamps, had longer dormancy and survived longer than those stored continuously in the dark. Moreover, the average dormancy of the microtubers stored under diffused light at 6 ± 1 °C was 28 weeks Gopal *et al*. (2004).

After a one year of storage period, all the tested accessions didn't show significant difference in mean microtuber germination ability neither at 4 °C nor at 1 °C temperatures (Table 3). All the selected accessions exhibited poor germination ability in both storage temperature levels. It was observed that though the microtubers showed poor germination ability, microtubers remained viable until six months. It indicated that induced dormancy was there. Ovono et al. (2010) found that, after 18 weeks microtubers storage at 25 °C, the germination in sterile

Table 3. Microtuber germination of selected accessions stored at 4 °C and 1 °C temperatures after one year

Accession	Mean microtuber germination %	
	At 4 °C	At 1 °C
11	1.8[a]	1.4[a]
22	1.6[a]	1.4[a]
29	1.7[a]	1.5[a]
117	2.2[a]	1.8[a]
129	1.2[a]	1.0[a]

** Mean values followed by the same letter are not significantly different at 0.05 probability*

compost was similar to that observed in *in-vitro* conditions: around 40 % germination after one week and 100 % after 8 weeks. In non sterile compost, the delay was more important but 100 % germination was also observed. They explained that even-though germination delayed in some tested media, it could be achieved 100 % in optimized media. Ovono et al. (2010) further highlighted that longer time period (22 weeks) was needed for microtuber germination when the microtubers were stored even at 18 °C temperature. Germination of the microtubers in *in-vivo* conditions was possible without special difficulties.

After one year storage period, microtuber categories > 7 mm and 5 - 7 mm of all selected accessions showed similar microtuber germination ability (Table 4). The similar phenomena in potato microtubers was reported where tuber categories ranging from 3 mm to > 1.0 mm could be conserved successfully at 1 °C temperature even for three months with 100 % survival (Edirisinghe *et al*. 2019). Onjo *et al*. (2001) highlighted that larger

Table 4. Effect of microtuber diameter on tuber germination which stored at 4 °C and 1 °C temperatures

Microtuber category (Diameter in mm)	Mean microtuber germination %	
	At 4 °C	At 1 °C
>7	3.68 a	3.20a
7-5	3.12a	2.40a
5-3	0	0
<3	0	0

** Mean values followed by the same letter are not significantly different at 0.05 probability level.*

microtubers of potato were able to produce a larger plants than plants from small potato microtubers. Large sized microtubers contained more food reserves; hence it could be able to sprout more easily. However, the dormant period was generally associated with a minimum endogenous metabolic activity, resulting in very little loss of storage reserve (Onjo *et al*. 2001). Vakis (1986) pointed out that microtubers which physiologically older or bigger in tuber size produced plantlets faster than physiologically young or smaller tubers. He also showed that storing of larger sized microtubers and their germinal vigor increased while the seeding was accelerated.

Potato microtubers which having larger size; > 1.5 cm to 2 cm in length was good for field planting Ovono et al. (2010). But all the tubers of those two groups remained viable without any color change even after one year storage period. Both categories gained similar result when it stored at 25 °C for 4.5 months. It revealed that even after 4.5 months or more than that its storage food reserves declined drastically and it starts shriveling fast. This study showed that the reduction of the microtuber storage temperature from 25 °C to 1 °C or 4 °C has reduced the microtuber germination percentage at one year conservation period.

Conclusion

Microtubers of four *D. alata* accessions and a *D. bulbifera* can be conserved at 25 °C for 4.5 months with 33 - 28 % microtuber germination. All selected accessions can be conserved at 1 °C or 4 °C temperatures for one year however, with ≤ 3.68 % microtuber germination. Microtuber having diameter of 5-7 mm and > 7 mm of all selected accessions can be conserved for 4.5 months at 25 °C with > 50 % microtuber sprouting percentage. But these two categories can be conserved both 1 °C, 4 °C temperatures for one year with low sprouting percentage without any germination differences. Microtuber having 3 - 5 mm and < 3 mm diameter of all selected accessions cannot be conserved at 1 °C, 4 °C or 25 °C temperatures. Further studies are needed to improve sprouting percentage of conserved *Dioscorea* microtubers at 1 °C or 4 °C temperatures prior to apply this as a conservation technique.

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