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Influence of Desiccation Time on Survival and Regeneration of Embryonic Axes of Groundnut (*Arachis hypogaea* L.) Immersed in Liquid Nitrogen

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ABSTRACT

Cryopreservation, the storage of biological materials in liquid nitrogen (LN), is a useful method for long term conservation of plant germplasm. This study was carried out with the objective of establishing an efficient desiccation technique for successful cryopreservation and recovery of embryonic axes of groundnut. Embryonic axes of four groundnut (*Arachis hypogaea* L.) genotypes were evaluated. The excised embryonic axes were dehydrated by air current of a laminar air flow cabinet for different duration (0, 1, 2, 3, 4 & 5 hrs) before being plunged in LN (−196˚C) and held for 1 hr. Samples were thawed in water bath at 40˚C for 2 min, thereafter cultured on MS medium supplemented with 15 mg/L BAP for recovery. Highest survival (96.67% - 100%) and shoot formation (91.67% - 96.67%) were obtained at an average moisture content of 17% after 4 - 5 hr desiccation. Among the genotypes evaluated, Samnut 22 and Samnut 23 recorded the highest survival and shoot formation. This technique therefore appears promising for cryopreservation of groundnut germplasm.

Keywords: Cryopreservation; Groundnut; Embryonic Axes; Desiccation

1. Introduction

Groundnut (*Arachis hypogaea* L.) is an important source of protein and edible oil in the world. Nigeria ranks third after India and China in terms of production [1]. Conservation of groundnut ensures availability of germplasm for future breeding needs, and seeds are the most preferred propagule used by seed bank curators for its storage. However, even under seed bank condition, long term storage of groundnut is not feasible as viability losses frequently occur [2]. This is because of its high oil content (45% - 50%), which makes it more perishable and prone to rapid loss of both quality and viability in storage [3]. For this reason, groundnut germplasm is maintained by planting every season in the Institute for Agricultural Research (IAR). This is not only laborious, time consuming and expensive, but also plants are exposed to the possible risk of pest, disease and environmental stresses. Newly improved genotypes of crops are fast replacing traditional genotypes or landraces which are often the source of diversity that breeders use for crop improvement. The need to conserve groundnut and other crop biodiversity therefore becomes imperative. Thus, cryopreservation should be considered as important complimentary strategy for *ex situ* conservation. Cryopreservation is used for long-term storage at ultralow temperature of −196˚C [4]. Cell division and metabolic activities are stopped when plants are exposed to ultra-low temperatures, allowing storage without alteration for an indefinite period of time [5]. Desiccation of excised embryos and embryonic axes is one of the most practicable techniques for cryopreservation [6]. Desiccation technique has been applied to a wide range of plant taxa which include embryonic axes of citrus [7,8] almond [9] and embryos of maize [10]. The present work is aimed at establishing an efficient desiccation technique for cryopreservation of embryonic axes of groundnut.

2. Materials and Methods

Seeds of four groundnut (*Arachis hypogaea* L.) genotypes were obtained from the groundnut breeding unit of IAR and used for this experiment. The seeds were surface sterilized by sequential treatment for 5 min in 70%

alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 - 3 drops of tween 20, rinsed thrice with sterile distilled water and immersed in 5% NaOCl plus 2 - 3 drops of tween 20 for 10 min with occasional stirring and washed three times with sterile distilled water. Thereafter seeds were soaked in sterile distilled water for 3 hr. Embryonic axes were excised and subjected to desiccation under the air current of a laminar flow cabinet for 0, 1, 2, 3, 4 and 5 hr exposure period. Moisture content (MC) was determined on fresh weight basis after drying in a 100˚C oven for 24 hr with 3 replicates of 10 embryonic axes per duration. Desiccated and nondesiccated (0) embryonic axes were placed in 2 ml sterile cryovial and directly immersed into liquid nitrogen (−196˚C) and held for 1 hr. Thawing took place in a water bath at 40˚C for 2 min. Embryonic axes were cultured individually in test tubes containing 10 ml of MS medium [11] supplemented with 15 mg/L 6-benzylaminopurine (BAP) and solidified with 8 g/L agar. Cultures were maintained in a growth chamber at 26˚C ± 2˚C under 16 hr light/8 hr dark photo period provided by white inflorescence. Three replicates of 10 embryonic axes were used per treatment. Survival was determined by the appearance of green color, increase in size, callusing and development of the root or shoot pole and expressed as a percentage of the total number of embryonic axes that survived within two weeks of culturing. While shoot formation was expressed as a percentage of the total number of embryonic axes forming shoots within one month of culturing. Data collected were subjected to analysis of variance (ANOVA) and means compared using Duncan's Multiple Range Test [12].

3. Results and Discussion

ANOVA for moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut is presented in **Table 1**. The main effects of desiccation and genotype were highly significant ($p \leq 0.01$) for all the characters. Two-way interaction between the desiccation time and genotype was also significant for all the characters.

Desiccation rates significantly influenced the extent of water loss of embryonic axes of groundnut, their survival and subsequent shoot formation after storage in liquid nitrogen (**Table 2**). Nondesiccated embryonic axes of groundnut failed to survive liquid nitrogen storage. This result corroborates that of Gagliardi, who observed non-survival of nondehydrated and cryopreserved embryonic axes of *Arachis* species [13]. Lack of germination of nondesiccated plant material has also been reported in other crops [14,15]. Similarly, embryonic axes desiccated for 1 hr also failed to survive the cryogenic treatment.

Table 1. Analysis of variance for moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut.

Source of variation	Degree of freedom	Mean Square		
		Moisture content (%)	Survival (%)	Shoot formation (%)
Desiccation(D)	5	1060.57**	131597.01**	116536.55**
Genotype (G)	3	99.60**	2539.94**	2410.05**
D x G	15	150.15**	3603.66**	2163.56**
Error	48	156.00**	1575.00**	489.99**

**$P \leq 0.01$.

Table 2. Effect of desiccation rate on moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut.

Treatment	Moisture content (%)	Survival (%)	Shoot formation (%)
Time of desiccation (hr) (D)			
0	27.33a	0.00d	0.00e
1	22.50b	0.00d	0.00e
2	18.25c	76.12c	59.35d
3	17.42c	83.75b	78.75c
4	17.25c	96.67a	91.67b
5	16.67c	100a	96.67a
SE±	0.74	2.34	1.30

Means followed by the same letter(s) within a column are not significantly different at $P < 0.05$ level of significance using DMRT.

Their nonsurvival could be attributed to the insufficient loss of moisture at this period which caused formation of lethal ice crystals that damage the cells during liquid nitrogen storage or thawing. Insufficient dehydration of the explants prior freezing may cause the formation of ice crystals during freezing or warming leading to the destruction of cellular structures and death of the explant [16]. Desiccation of embryonic axes from 2 hr to 5 hr rapidly caused significant loss of moisture content. This greatly improved the survival and shoot formation of cryopreserved embryonic axes. The highest survival (96.67% - 100%) and subsequent shoot formation (91.67% - 96.67%) was obtained at an average moisture content of 17% after 4 - 5 hr desiccation rates. [17] reported 100% germinability of embryonic axes when moisture content was reduced from 25% to 8.5% which was achieved at 2.5 hr desiccation time. While [13], reported 80% shoot development at 18% moisture content after 1 hr desiccation rate in *Arachis* species. The improved survival and shoot formation with decreasing moisture content could be due to increased accumulation of sugars during drying. As it is possible that accumulation of sugars may serve to maintain cellular integrity by osmotically decreasing cell volume, or act directly to protect by stabilization of membranes [18]. Another possibility is the probable accumulation of abscisic acid (ABA) in the desiccated embryos. ABA is reported to promote desiccation tolerance in mature embryo through the synthesis of late embryogenesis abundant (LEA) proteins encoded by mRNA [19], ABA has also been implicated in cold acclimation in plants [20].

The moisture content of excised embryonic axes is the most critical factor influencing the success of cryopreservation using desiccation protocol. This to some extent is influenced by the desiccation rate. Results from correlation studies **(Table 3)** indicate that moisture content was negatively correlated with survival and shoot formation of embryonic axes after cryopreservation ($r = -0.41$ and -0.35, respectively). This implies that any significant loss of moisture will greatly improve survival and subsequent shoot formation of cryopreserved embryonic axes of groundnut. Therefore samples to be cryopreserved must be sufficiently dehydrated to avoid lethal intracellular freezing. [21] reported that, survival and emergence of post-thaw embryos were closely related to their moisture contents prior to freezing.

Results obtained from this study showed a significant difference among the genotypes evaluated (**Figure 1**). Samnut 22 embryonic axes recorded the highest moisture content, which was statistically the same as that of Samnut 10 and Samnut 21. This was followed by Samnut 23. Similarly, significant survival and shoot formation of

Table 3. Correlation coefficients between moisture content, survival and shoot formation of groundnut as influenced by desiccation.

	Moisture content (%)	Survival (%)	Shoot formation (%)
Moisture content (%)	1.00		
Survival (%)	−0.41	1.00	
Shoot formation (%)	−0.35	0.98	1.00

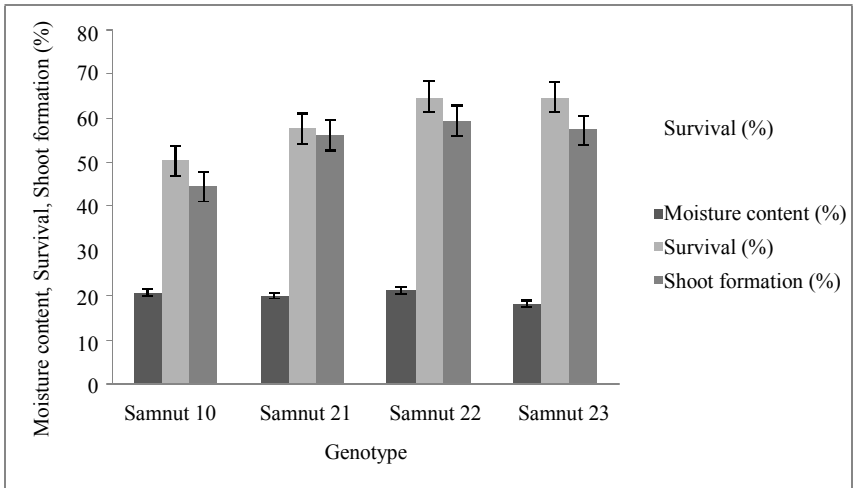

Figure 1. Influence of genotype on moisture content, survival and shoot formation of cryopreserved embryonic axes of groundnut.

cryopreserved embryonic axes was observed among the genotypes. Samnut 22 and Samnut 23 which are at par with each other had the highest survival and shoot formation followed by Samnut 21. While Samnut 10 had a relatively lower survival and shoot formation. The observed differences among the genotypes could be due to genotypic influence, as tissue culture response in groundnut is strongly influenced by the plant genotype [22,23]. Genotypic influence has also been reported in cryopreserved embryonic axes of maize [24]. There was a significant interaction between the genotypes and time of desiccation for moisture content of cryopreserved embryonic axes of groundnut. Samnut 10 had the highest moisture content prior to desiccation which was comparable to that recorded by Samnut 22, followed by Samnut 21 and Samnut 23. At 1 hr desiccation time Samnut 10 significantly had higher moisture content compare to the other genotypes, from 2 hr to 5 hr desiccation time all the genotypes recorded comparable low moisture content (**Table 4**). The genotype x desiccation interaction on survival clearly indicated that embryonic axes of all the genotypes did not survive cryopreservation when none desiccated and when desiccated for 1 hr (**Table 5**). However, at 2 hr and 3 hr desiccation time Samnut 22 and Samnut 23 comparably recorded the highest survival rates compare to the other genotypes. While from 4 hr to 5 hr desiccation time, all the genotypes comparably recorded very high survival rates. Effect of genotype x desiccation interaction on shoot formation of cryopreserved embryonic axes was significant (**Table 6**). Samnut 21, Samnut 22 and Samnut 23 comparably produce more shoots than Samnut 10 when desiccated for 2 hr. At 3 hr desiccation time Samnut 22 and Samnut 23 had higher shoot formation than Samnut 21 followed by Samnut 10. All the groundnut genotypes comparably recorded high shoot formation at 4 hr desiccation time, with the exception of Samnut 10 which significantly recorded the least shoot formation. However, at 5 hr desiccation time, all the groundnut genotypes recorded comparably high shoot formation after cryopreservation. This suggests that the effect of time of desiccation was not uniform over the different genotypes.

Table 4. Effect of genotype x desiccation interaction on the moisture content of cryopreserved embryonic axes of groundnut.

Genotype	Treatment level: Time of desiccation					
	0	1	2	3	4	5
Samnut 10	31.33a	24.67b	18.67c	17.67c	16.00c	15.67c
Samnut 21	26.00b	22.00bc	18.33c	17.00c	17.67c	18.33c
Samnut 22	27.67ab	26.00b	19.00c	18.67c	18.00c	17.00c
Samnut 23	24.33b	17.33c	17.00c	16.33c	17.33c	15.67c

Means followed by the same letter(s) are not significantly different at P < 0.05 level of significance using DMRT.

Table 5. Effect of genotype x desiccation interaction on the survival of cryopreserved embryonic axes of groundnut.

Genotype	Treatment level: Time of desiccation					
	0	1	2	3	4	5
Samnut 10	0e	0e	53.83d	60cd	86.67ab	100a
Samnut 21	0e	0e	71.43c	75bc	100a	100a
Samnut 22	0e	0e	88.89ab	100a	100a	100a
Samnut 23	0e	0e	88.33ab	100a	100a	100a

Means followed by the same letter(s) are not significantly different at P < 0.05 level of significance using DMRT.

Table 6. Effect of genotype x desiccation interaction on the shoot formation of cryopreserved embryonic axes of groundnut.

Genotype	Treatment level: Time of desiccation					
	0	1	2	3	4	5
Samnut 10	0f	0f	47.50e	60.00cd	70.00bc	90a
Samnut 21	0f	0f	62.50cd	75b	100a	100a
Samnut 22	0f	0f	66.67bcd	90a	100a	100a
Samnut 23	0f	0f	60.71cd	90a	96.67a	96.67a

Means followed by the same letter(s) are not significantly different at P < 0.05 level of significance using DMRT.

The survived and regenerated shoots after 4 weeks were rooted on MS medium supplemented with 1mg/L 1-naphthaleineacetic acid (NAA) [25]. These were then transferred to potty soil (riverside sand) for hardening. No morphological abnormalities were observed in the plants developed from cryopreserved embryonic axes by desiccation (**Figures 2-5**).

4. Conclusions

Results obtained in this study indicate significant genotypic differences in response to the desiccation rates.

However, in view of the very high survival (96.67% - 100%) and shoot regeneration (91.67% - 96.67%) obtained in the groundnut genotypes, it can be concluded that cryopreservation using desiccation protocol can be successfully applied to groundnut, and the provided moisture content is reduced to about 17%. Hence, this

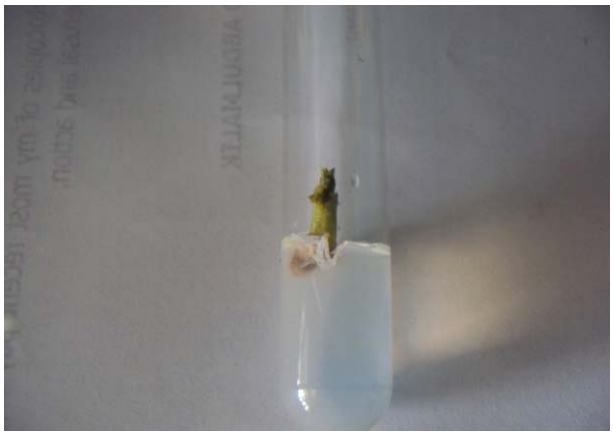

Figure 2. Microshoot regeneration from cryopreserved embryonic axes on MS + 15 mg/L BAP after 2 wk culture.

Figure 3. Microshoot on MS + 15 mg/L BAP after 4 wk culture.

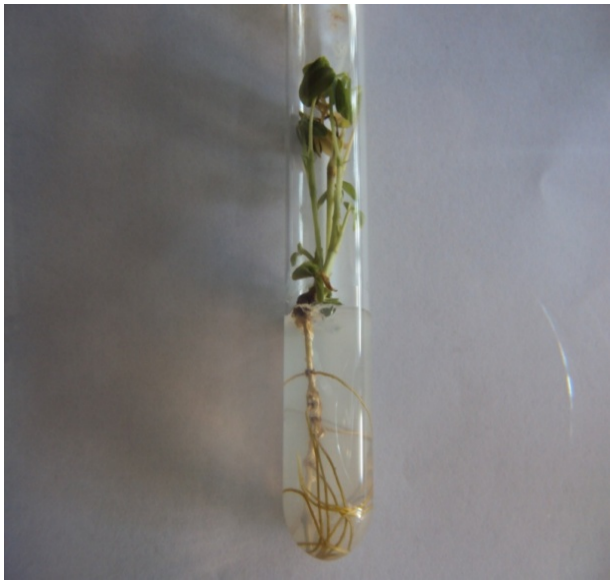

Figure 4. Regenerated Plantlet rooted on MS + 1 mg/L NAA.

Figure 5. A potted plant in green house.

technique could be routinely employed for conservation of groundnut germplasm.

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