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SLOW GROWTH *IN VITRO* CONSERVATION OF GROUNDNUT (*Arachis hypogaea* L.)

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ABSTRACT

In vitro conservation techniques such as slow growth is now routinely used to compliment conventional conservation approaches. Slow growth procedures provide short to medium term storage options, and offer several advantages such as: free from genetic erosion and convenient for international distribution. The present study is aimed at establishing a slow growth *in vitro* conservation protocol for Nigerian groundnut varieties. The experiment was conducted with four groundnut varieties: SAMNUT 10, SAMNUT 21, SAMNUT 22 and SAMNUT 23, four conservation media: Murashige and Skoog (MS) media supplemented with different concentrations of sucrose (30, 20,15,10g/L) in combination with different concentrations of mannitol (10,15, 20g/L) and maintained for four different conservation periods: 3, 6, 9 and 12 months. The experiment was arranged as 4 x 4 x 4 factorial experiment in a completely randomized design of three replicates. *In vitro* regenerated shoots of 2cm length were cultured on slow growth media. Results obtained from the study show that maximum survival (33.1%) was observed in SAMNUT 23. While MS medium supplemented with 10g/L sucrose and 20g/L mannitol effectively reduced shoot growth and produced the highest survival (74%) after 3months conservation period. This medium could therefore be used for the medium-term conservation of the groundnut varieties.

Keywords*: in vitro conservation, slow growth, mannitol, sucrose, groundnut.*

INTRODUCTION

Groundnut is an important crop worldwide for the production of oil and protein from the seed. Groundnut production is hampered by multitude of biotic and abiotic stresses, of which there are few improved varieties that are resistant to these stresses and this is mainly due to the narrow genetic base of the cultivated groundnut (Kochert *et al*., 1991). Securing existing and novel groundnut germplasm is essential for future improvement programs. Conventionally, groundnut germplasm is conserved in gene banks. However, even under gene bank condition groundnut seeds cannot be stored for long like other true orthodox seeds owing to its high lipid content and thin seed coat. Advances in biotechnology now provide new options for improved conservation of plant genetic resources using *in vitro* culture techniques such as slow growth for short- and medium-term conservation and cryopreservation for long-term conservation. Cryopreservation is the storage of plant material in liquid nitrogen at temperature of -196^0C, at which all the cells are in a state of suspended animation (Engelmann, 2004). In slow growth technique, the plant material is subjected to growth retardation conditions that slow down plant growth and development *in vitro*. This includes growth under low temperatures, reduced light intensity, and the use of growth retardants such as abscisic acid (ABA) and by the addition of osmotic agents such as sucrose, mannitol or sorbitol in the culture medium (Hassan *et al*., 2014; Al-Abdallat *et al*., 2017; Zayova *et al*., 2017; Rodrigues *et al*., 2018). Slow growth technique is well established and applied to a wide range of plant species with high recovery growth and maintenance of genetic stability (Hassan *et al*., 2014; Gianní and Sottile, 2015). Slow growth procedures also offer several advantages such as; the possibility for the establishment of core collection with long-term gene banks, not expensive when *in vitro* facilities are already present, easy and convenient for international distribution (Malaurie *et al*., 1998). Slow growth techniques could be considered as important complementary strategies for efficient conservation of groundnut germplasm. The objective of this study was to establish a slow growth *in vitro* conservation protocol for Nigerian groundnut varieties that when applied alongside with the conventional seed bank approach will improve the conservation of the crop.

MATERIALS AND METHODS

Experimental site

The study was conducted at the Biotechnology laboratory, Department of Plant Science Institute for Agricultural Research (IAR), Ahmadu Bello University Zaria.

Experimental design

The experiment was arranged as 4 x 4 x 4 factorial experiment in a completely randomized design of three replicates.

Plant materials

Four (4) IAR improved varieties of groundnut; SAMNUT 10, SAMNUT 21, SAMNUT 22 and SAMNUT 23 were used in the study.

Surface sterilization procedure

The seeds were surface sterilized using double sterilization sequence under the laminar flow hood. The first sterilization sequence involves treatment in 70% alcohol for 5min, followed by 10% Chlorox (commercial bleach containing 3.5% NaOCl)) plus 2-3 drops of Tween 20 for 20min with occasional stirring. The seeds were then rinsed thrice with sterile distilled water. This was followed by the second sterilization sequence when the seeds were immersed in 5% Chlorox plus 2-3 drops of Tween 20 for 10min with occasional stirring and then rinsing thrice with sterile distilled water.

Culture initiation and conservation procedure

Embryonic axes were dissected from sterile seeds and cultured on Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 15mg/L 6-benzylaminopurine (BAP) and solidified with 8g/L agar. *In vitro* regenerated shoots of 2cm length were subcultured on the slow growth media. The slow growth media consisted of MS media supplemented with different concentrations of sucrose (30, 20,15,10g/L) in combination with different concentration of mannitol (10,15, 20g/L) and maintained for different conservation periods (3, 6, 9 and 12 months). Media were adjusted to pH 5.8 before autoclaving at 121^0C and 15psi air pressure for 15 min. The cultures were maintained at 26±2ºC. Data was recorded at the end of each conservation period on survival and shoot length. The survived shoots were cultured on recovery medium (MS medium supplemented with 15mg/L BAP) and cultures were incubated under normal growth conditions.

Statistical analysis

Data collected were subjected to analysis of variance using the general linear model (procedure of the statistical analysis system (SAS Institute Inc., 1990). Means were compared using Duncan's multiple range test (DMRT) at 0.05 probability level.

RESULTS

Effect of Sucrose and Mannitol Concentrations, Varieties and Conservation Period on Survival Rates of Slow Growth Maintained Cultures.

The effect of different sucrose and mannitol concentrations, on survival was observed to be significant ($p \leq 0.05$) as shown in Table 1. Results revealed that the highest mean value of survival rate (33.8%) was obtained on MS medium supplemented with 10g/L sucrose and 20g/L mannitol, followed by survival rate (30.6%) recorded from medium supplemented with 20g/L sucrose and 10g/L mannitol and survival rate (29.4%) recorded by medium supplemented with 15g/L sucrose and 15g/L mannitol which were statistically similar. The lowest mean value of survival percentage (25.0%) was observed in the control medium. As for the effect of variety on survival of cultures, results in Table 1 showed that the highest survival (33.1%) was recorded by SAMNUT 23, followed by SAMNUT 22 (29.4%) and SAMNUT 10 (30.0%) which were statistically the same. The lowest survival rate (26.3%) was recorded by SAMNUT 21. With regard to the effect of different conservation periods, the highest survival (73.8%) was obtained from cultures conserved for 3 months, followed by survival rates (45.0%) obtained from 6 months conservation period. Cultures maintained for 9 months and 12 months, failed to survive (Table 1: Figure 1).

Table 1: Effect of sucrose and mannitol concentrations, varieties and conservation period on survival rates of slow growth maintained cultures.

Treatment	Survival (%)
Medium supplements (g/L) (**M**)	
MS + 30 sucrose(Control)	25.0c
MS + 20 sucrose+10 mannitol	30.6b
MS + 15 sucrose+15 mannitol	29.4b
MS + 10 sucrose+20 mannitol	33.8a
SE±	1.14
Variety (**V**)	
SAMNUT 10	30.0b
SAMNUT 21	26.3c
SAMNUT 22	29.4b
SAMNUT 23	33.1a
SE±	1.14
Period (months) (**P**)	
3	73.8a
6	45.0b
9	0.0c
12	0.0c
SE±	1.14
M x V x P	NS
CV (%)	18.83

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. NS non significant, NS not significant.

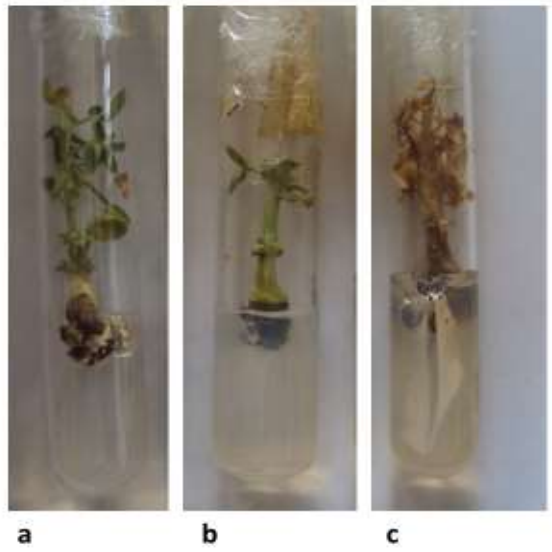

Fig. 1: Effect of conservation period on survival of groundnut (a) plant after 3months storage (b) plant after 6month storage (c) plant after 9months storage

Effect of Sucrose and Mannitol Concentration on Shoot Height

Shoot height was significantly ($p \leq 0.05$) retarded with decreasing concentration of sucrose and increasing concentration of mannitol in the conservation medium (Table 2; Figure 2). The highest shoot height (7.83cm) was obtained in the control medium, followed by shoot height (5.25cm) obtained from medium supplemented with 20g/L sucrose and 10g/L mannitol and shoot height (4.16cm) obtained from medium supplemented with 15g/L sucrose and 15g/L mannitol. Whereas medium supplemented with 10g/L sucrose and 20g/L mannitol recorded the lowest shoot height (3.03cm).

Table 2: Effect of sucrose and mannitol concentration on shoot height

Treatment	Shoot height
Medium supplements (g/L)	
MS + 30 sucrose(Control)	7.8a
MS + 20 sucrose+10 mannitol	5.3b
MS + 15 sucrose+15 mannitol	4.2c
MS + 10 sucrose+20 mannitol	3.0d
SE±	0.39
CV (%)	26.53

Means followed by the same letter(s) are not significantly different at $p \leq 0.05$ level of significance using DMRT. NS non significant, NS not significant.

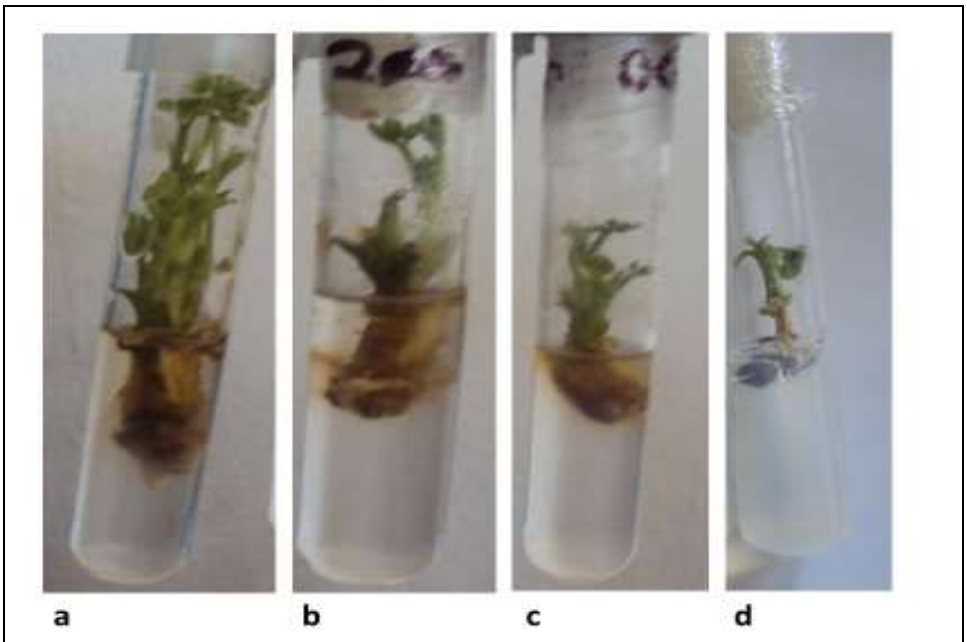

Fig. 2: Effect of sucrose & mannitol on shoot height of groundnut (a) control (b) microshoot on MS + 20mg/L sucrose+ 10mg/L mannitol(c) microshoot on MS + 15mg/L sucrose+ 15mg/L mannitol (d) microshoot on MS + 10mg/L sucrose+ 20mg/L mannitol

DISCUSSION

Many plant species have been conserved successfully using *in vitro* conservation techniques (Al-Abdallat *et al*., 2017; Zayova *et al*., 2017; Rodrigues *et al*., 2018) by the addition of osmotic agents such as sucrose, mannitol or sorbitol in the culture medium (Charoensub and Phansiri, 2004; Hassan *et al*., 2014). The maintenance of shoot cultures is obtained through the slowing down of cell metabolism, as a consequence of shoot growth. The use of osmotically active compounds, such as sucrose and mannitol can interfere on shoot growth and influence markedly the maximum storage time (Scherwinski-Pereira *et al*., 2010). In the present study sucrose and mannitol were used to induce slow growth in groundnut varieties. It was revealed from results that maximum survival (33.8%) was obtained on MS medium supplemented with 10g/L sucrose and 20g/L mannitol while the lowest survival (25.0%) was observed in the control medium. This reaffirms that sucrose and mannitol are essential for survival of cultures under *in vitro* conservation. Sarkar and Naik (1998) reported that 20 or 40 g/L mannitol in combination with sucrose could enhance survival of *in vitro* conservation of potato. Similarly, Charoensub and Phansiri (2004) also reported that the addition of 20 g/L mannitol to leadwort culture medium prolonged subculture time to 8 months. However, Slow growth storage methods are extremely variable and genotype dependent (Wilkins *et al*. (1988). In the present study survival of cultures after the slow growth treatment differed with varieties, the highest survival (33.1%) was recorded by SAMNUT 23, followed by SAMNUT 22 (29.4%). The observed difference among the varieties could be attributed to their differential genomic constitution. This finding is in agreement with that of Gianní and Sottile (2015) who reported the existence of genotypic difference in terms of survival in plum genotypes under slow growth. The effect of different conservation periods on survival rates of microshoots indicated that maximum survival (73.8%) was obtained from cultures conserved for 3 months. Survival of cultures declined drastically to 45.0% after 6 months conservation period. Cultures maintained for 9 months and 12 months, however, failed to survive. The observed trend could be attributed to the decrease in nutrients and increased in osmotic stress. Lack of survival could also be due the depletion of oxygen concentration in the nutrient medium which could have led to anaerobic respiration. As plant tissues when subjected to hypoxic (low) or anoxic (zero) concentration of ambient oxygen, are often forced to carry out anaerobic respiration. This could lead to lactate accumulation which promotes acidification of the cytosol (Taiz and Zeiger, 2003). Maximum survival and healthy shoot cultures in the first 3 months of conservation and decreased in survival after 6, 9 to 12 months have been reported in grape (Hassan *et al*., 2014) and in globe artichoke (Shawky and Aly, 2007). Osmotic regulators act as growth retardants by causing osmotic stress to the material under conservation. When added to the culture medium, these carbohydrates reduce the hydric potential and restrict the water availability to the explant (Shibli *et al.* 2006) thereby reducing the optimal turgor pressure needed for cell division and inhibit growth (Tahtamouni *et al*., 2001). In the present study shoot length was significantly retarded with decreasing concentration of sucrose and increasing concentration of mannitol in the conservation medium. Microshoots maintained on medium supplemented with 10g/L sucrose and 20g/L mannitol recorded the lowest shoot length (3.0cm). The reduction in growth could be due to increased osmotic stress imposed by the osmoticum in the culture medium and reduced nutrients. Minoo *et al.* (2006) also observed significant decrease in shoot length of vanilla with increase concentration of mannitol and reduced sucrose in culture medium.

CONCLUSION

Results obtained from the slow growth experiment show that MS medium supplemented with 10g/L sucrose and 20g/L mannitol effectively reduced shoot growth and produced the highest survival (74%) after 3months conservation period of the groundnut varieties. This medium could therefore be used for the medium-term conservation of these varieties with subculturing intervals of three months.

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