academicJournals

Vol. 13(2), pp. 280-285, 8 January, 2014
DOI: 10.5897/AJB2013.13452
ISSN 1684-5315 ©2014 Academic Journals
http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Cryopreservation of embryonic axes of groundnut (*Arachis hypogaea* L.) by vitrification

M. M. Abdulmalik*, I. S. Usman, J. D. Olarewaju and D. A. Aba

Department of Plant Science, P.M.B.1044, Ahmadu Bello University, Zaria, Kaduna State, Nigeria.

Accepted 11 December, 2013

An efficient cryopreservation protocol was developed for groundnut embryonic axes using vitrification technique. Embryonic axes obtained from seeds of four groundnut genotypes were dehydrated in Plant Vitrification Solution (PVS2) solution for different durations (0, 1, 2, 3, 4 and 5 h) before plunged into liquid nitrogen and held for 1 h. Survival and shoot formation of cryopreserved embryonic axes were significantly influenced by the dehydration duration with embryonic axes treated for 2 h recording the highest survival (70%) and shoot formation (65%). Among the groundnut genotypes evaluated, Samnut 22 and 23 gave the highest survival (74.44 and 75.56%) and shoot formation (72.22 and 72.78%) after cryopreservation.

Key words: Cryopreservation, embryonic axes, groundnut, vitrification.

INTRODUCTION

Groundnut (*Arachis hypogaea* L.) is an important crop worldwide. It is cultivated in at least 100 countries of which China (40.9%), India (14%), Nigeria (7.44%) and the United States (7.41%) are the largest producers. World production of the crop is about 41.27 million metric tonnes, with an average yield of 1.68 tons/ha (FAOSTAT, 2012). Almost every part of the groundnut plant is economically important. The seed contain high quality edible oil (44 to 52%), easily digestible protein (26 to 28%) and carbohydrate (20%), besides vitamin (E, K, B1 and B3) mineral and dietary fiber. Haulms (leaves and stalk) are utilized as fodders while the cake, formed after the oil extraction is a high protein animal feed. The shells are used as fuel, filler in feed industry and in making cardboards. Being a legume with root nodules, it enriches the soil by fixing atmospheric nitrogen, thereby contributing to soil fertility (Waliyar et al., 2007). Groundnut germplasm is conventionally stored in gene banks and seeds are the preferred propagule used. However, due to their high lipid content and thin seed coat they cannot tolerate the gene bank conditions for longer periods like other true orthodox seeds. This led to the suggestion that groundnut should be considered as suborthodox (Vazquez-Yanes and Arechiga, 1996; Gagliardi et al., 2000).

Seeds of some *Arachis* species have been reported to lose viability after 2 to 3 years of storage (Roberts and Ellis, 1989; Dunbar et al., 1993). Studies on groundnut seeds viability in Nigeria have revealed that 75% of all the evaluated lines lost viability after 2 years of storage (Alarou, 2006). This therefore implies that long term conservation of groundnut seeds using conventional techniques is impossible. Cryopreservation is becoming a popular technique used by gene bank curators for storage of challenging plant germplasm. Cryopreser-vation is the storage of biological material in liquid nitrogen (LN) at ultra low temperature (-196°C). At this temperature, all cellular divisions and metabolic processes are stopped which allows conservation for an unlimited period of time (Engelmann, 2004). Cryopreservation benefits include small space requirements, low maintenance costs, and only a modest number of replicates is required to conserve

*Corresponding author. E-mail: uwa6474@yahoo.com. Tel: 00985614313853. Fax: 00985612254050.

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

Source of variation	Degree of freedom	Mean square	
		Survival (%)	Shoot formation (%)
Dehydration in PVS2	5	3839.17**	3648.06**
Genotype (G)	3	15586.57**	14871.76**
PVS2 × G	15	615.46**	653.98**
Error	48	66.67**	48.96**

**P≤0.01.

a plant effectively (Shibli and Al-Juboory, 2000). It also has an added advantage of long-term storage without subjecting to frequent subculturing which is known to induce somaclonal variation (Jain, 2011). Cejas et al. (2013) reported genetic stability of cryopreserved plants at both phenotypic and molecular levels over time. Recently, cryopreservation has been applied in the elimination of virus and bacteria pathogens (Ding et al., 2008; Helliot et al., 2002).

The most popular cryopreservation techniques in use are the vitrification-based; they include vitrification, desiccation, encapsulation-dehydration and encapsulation-vitrification. Cryopreservation by vitrification has an advantage of being simple, having greater survival rate and applicable to a wide range of plant material. However, the utilization of cryopreservation requires an efficient and reproducible protocol that will ensure high survival and regeneration. Hence, this study was carried out with the objective of establishing an efficient cryopreservation protocol for groundnut using vitrification.

MATERIALS AND METHODS

Seeds of four groundnut genotypes (SAMNUT 10, 21, 22 and 23) were obtained from the groundnut breeding unit of the Institute for Agricultural Research, Ahmadu Bello University, Zaria. The seeds were surface sterilized by sequential treatment for 5 min in 70% alcohol, 20 min in 10% NaOCl (commercial bleach) plus 2 to 3 drops of tween 20, rinsed thrice with sterile distilled water and immersed in 5% NaOCl plus 2 to 3 drops of tween 20 for 10 min with occasional stirring and rinsed thrice with sterile distilled water. Thereafter, seeds were soaked in sterile distilled water for 3 h. Embryonic axes were excised from the seeds and precultured on solidified Murashige and Skoog (MS) medium (Murashige and Skoog, 1962) supplemented with 0.3 M sucrose for 24 h. Precultured embryonic axes were then treated with a loading solution (2 M glycerol + 0.4 M sucrose dissolved in MS medium) for 15 min at 25°C. Treated embryonic axes were transferred to 2 ml cryovials and 1 ml PVS2 [30%(w/v) glycerol, 15%(w/v) ethylene glycol and 15% (w/v) dimethylsulfoxide (DMSO) in MS medium with 0.4 M sucrose] was added with a micropipette and dehydrated for different duration (0, 1, 2, 3, 4 and 5 h) at 25°C before directly plunged into LN and held for 1 h. After storing, cryovials were rapidly warmed in a water bath at 40°C for 2 min. PVS2 solution was drained from cryovials, replaced twice with 1 ml of unloading solution (1.2 M sucrose dissolved in MS medium), and held for 10 min. Cryopreserved embryonic axes were then cultured on MS medium supplemented with 15 mg/L benzylaminopurine (BAP) for recovery under 16 h photoperiod at room temperature. Data were collected on survival and shoot formation. 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 embryonic axes that survived within two weeks of culturing.

Shoot formation was expressed as percentage of embryonic axes forming shoots within one month. Treatments were arranged in a completely randomized design (CRD) with ten embryonic axes per treatment and these were replicated three times. Data collected were analyzed using the general linear model (GLM) procedure of the statistical analysis system (SAS Institute Inc., 1990). Means were compared using Duncan's multiple range test (Duncan, 1955) at 0.05 probability level.

RESULTS

To determine the optimum time of exposure to PVS2, embryonic axes of groundnut were dehydrated with PVS2 for different duration prior to a plunge into LN. Dehydration duration in PVS2 significantly ($P<0.05$) affected the rates of survival and subsequent shoot formation of the cryopreserved embryonic axes (Tables 1 and 2). Very low survival and shoot formation was observed when embryonic axes were treated in PVS2 for 1 h. The highest survival (70%) and shoot formation (65%) was obtained when embryonic axes were dehydrated in PVS2 for 2 h. Increasing the dehydration time from 3 h upwards significantly ($P<0.05$) decreased the rate of survival and shoot formation. Significant genotypic differences were also observed among the genotypes in response to the PVS2 treatment (Table 2). SAMNUT 22 and 23 had the highest survival (74.44 and 75.56%) and shoot formation (72.22 and 72.78%), followed by SAMNUT 10; while SAMNUT 21 recorded the lowest survival and shoot formation.

Significant ($P<0.05$) interactions were observed between genotype and dehydration time in PVS2 on survival and shoot formation rate (Table 1). Keeping the genotype constant and varying the dehydration time in PVS2 from 1 to 2 h, there was a significant increase in the survival and shoot formation rates in all the genotypes. However, further increase in time resulted in a decline in the survival and shoot formation rates with the exception of SAMNUT 22 which significantly maintained higher survival and shoot formation rates. Individual genotype interaction with the dehydration time in PVS2 indicated that there was a good interaction

Table 2. Effect of dehydration time in PVS2 on survival and shoot formation of embryonic axes of groundnut.

Treatment	Survival (%)	Shoot formation (%)
Dehydration in PVS2 (h)		
0	73.33[a]	73.33[a]
1	45.83[b]	40.00[d]
2	70.00[a]	65.00[b]
3	46.67[b]	45.83[c]
4	46.67[b]	46.67[c]
5	25.00[c]	25.00e
SE±	2.55	2.86
CV (%)	15.93	14.19
Genotype		
Samnut 10	40.56[b]	38.89[b]
Samnut 21	14.44[c]	13.33[c]
Samnut 22	74.44[a]	72.22[a]
Samnut 23	75.56[a]	72.78[a]
SE±	2.08	2.33
CV (%)	15.93	14.19

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

Table 3. Effect of genotype × dehydration in PVS2 interaction on the survival of cryopreserved embryonic axes of groundnut.

Genotype	Treatment level					
	Dehydration in PVS2 (h)					
	0	1	2	3	4	5
SAMNUT 10	66.67[c]	43.33[d]	80.00[b]	46.67[d]	6.67[f]	0.00[f]
SAMNUT 21	46.67[d]	6.67[f]	26.67[e]	6.67[f]	0.00[f]	0.00[f]
SAMNUT 22	93.33[a]	66.67[c]	86.67[ab]	90.00[a]	60.00[c]	50.00[d]
SAMNUT 23	86.67[ab]	66.67[c]	86.67[ab]	83.33[b]	80.00[b]	50.00[d]

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

Table 4. Effect of genotype × dehydration in PVS2 interaction on the shoot formation of cryopreserved embryonic axes of groundnut.

Genotype	Treatment level					
	Dehydration in PVS2 (h)					
	0	1	2	3	4	5
SAMNUT 10	66.67[d]	33.33[g]	80.00[c]	46.67[f]	6.67[j]	0.00[j]
SAMNUT 21	46.67[f]	6.67i	20.00[h]	6.67[j]	0.00[j]	0.00[j]
SAMNUT 22	93.33[a]	60.00[e]	80.00[c]	90.00[ab]	60.00[e]	50.00[f]
SAMNUT 23	86.67[b]	60.00[e]	80.00[c]	80.00[c]	80.00[c]	50.00[f]

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

between SAMNUT 22 and 23 up to 3 h dehydration time. However, these genotypes were not different from SAMNUT 10 in both survival and shoot formation rates. SAMNUT 21 consistently recorded the lowest survival and shoot formation rates throughout the dehydration duration in PVS2 (Tables 3 and 4). The cryopreserved

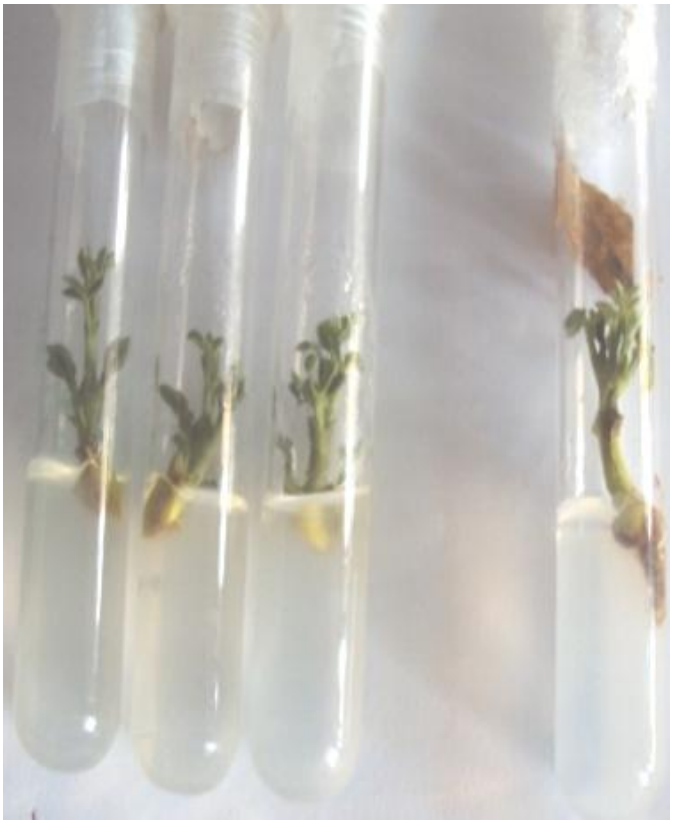

Figure 1. Recovered microshoots after 4 weeks on MS media + 15 mg/L BAP. Microshoots from cryopreserved embryonic axes (a), microshoots from non cryopreserved embryonic axes (b).

embryonic axes resume growth within one week of culturing on recovery medium and developed shoots within three to four weeks without intermediary callus formation. There were no apparent morphological abnormalities observed in the regenerated microshoots and resultant plantlets (Figures 1 and 2).

DISCUSSION

One of the key to successful cryopreservation by vitrification is the careful control of dehydration and prevention of injury by chemical toxicity. Optimization of the time of exposure to PVS2 is important for producing a high level of shoot formation after vitrification. In this study, we were able to obtain the highest survival and shoot formation with embryonic axes dehydrated with PVS2 for 2 h. Ozudogru et al. (2009) also observed very high survival rates at 2 h time of dehydration in PVS2 for embryonic axes of groundnut. Very high survivals were also reported at 1 to 2 h for embryonic axes of groundnut (Gaglardi et al., 2002). The high survival and shoot formation reported here could be attributed to sufficient dehydration. Since only after sufficient dehydration will the cell content be able to vitrify upon rapid cooling in liquid nitrogen without forming lethal ice crystals and hence ensuring high survival. According to Volk and Walters (2006), three mechanisms by which PVS2 aids cryoprotection are by replacing cellular water, changing the freezing behaviour of water remaining in cells and impeding water loss during air drying. In addition to their physical protective properties, cryoprotectants also impart additional protection against cryoinjury as they can stabilize proteins and membranes and act as antioxidants (Fuller, 2004).

The poor survival and shoot formation observed at 1 h time of exposure to PVS2 could be attributed to the insufficient dehydration of the embryonic axes. While the drastic decline in both survival and shoot formation rates observed from 3 to 5 h dehydration period could be due to the toxicity of the PVS2. Chemical toxicity of PVS2 usually increases with time and causes a decrease in survival rates (Yamuna et al., 2007). The poor performance of SAMNUT 10 and 21 compared with the other genotypes could be attributed to the sensitivity of these genotypes to the PVS2 treatment. Takagi (2000) reported

Figure 2. Regenerated plantlets flowering. Plantlet from cryopreserved embryonic axes (a); plantlet from non cryopreserved embryonic axes (b).

that sensitivity to dehydration by vitrification solution varies among species and even within cultivars. Genotypic influence has been reported in vitrified embryonic axes of maize (Usman and Abdulmalik, 2010) and shoots tips of grape (Matsumoto and Sakai, 2003). The significant interaction between genotype and dehydration time further confirms that dehydration time in PVS2 is genotype-dependent. The high post thaw survival and shoot formation recorded in this study could also have been enhanced by the preculturing of the embryonic axes on high sucrose medium and treating with loading solution prior to exposure in PVS2.

Matsumoto et al. (1994, 1995) reported the positive effect of preculturing on high sucrose media and of loading treatment on post thaw survival.

Conclusion

Results obtained show that exposure time in PVS2 is very critical to survival and subsequent shoot formation of cryopreserved embryonic axes of groundnut. Most of the groundnut genotypes recorded very high survival and shoot formation at 2 h exposure time in PVS2. This therefore indicates the potential of cryopreservation by vitrification for conservation of groundnut.

REFERENCES

Alarou AD (2006). Effect of storage on viability of groundnut lines. Unpublished B.Agric. Project. Faculty of Agriculture Ahmadu Bello Uni. Zaria, Nigeria.

Cejas I, Méndez R, Villalobos A, Palau F, Aragón C, Engelmann F, Carputo D, Aversano R, Martínez ME, Lorenzo JC (2013). Phenotypic and Molecular Characterization of *Phaseolus vulgaris* Plants from Non-Cryopreserved and Cryopreserved Seeds. Am. J. Plant Sci. 4:844-849.

Ding F, Jin SX, Hong N, Zhong Y, Cao Q, Yi GJ, Wang GP (2008). Vitrification-cryopreservation, an efficient method for eliminating *Candidatus Liberobacter asiaticus*, the citrus Huanglongbing pathogen, from *in vitro* adult shoot tips. Plant Cell Rep. 27:241-250.

Dunbar KB, Pittman RN, Morris JB (1993). *In vitro* culture of embryonic axes from Arachis species for germplasm survival. J. Seed Technol. 17:1-8.

Duncan DB (1955). Multiple Range and Multiple F-test. Biometrics 11:1-42.

Engelmann F (2004). Plant cryopreservation: progress and prospect. *In vtro* Cell Dev Biol Plant. 40:427-433.
FAOSTAT (2012). http://faostat.fao.org/default.aspx
Fuller BJ (2004). Cryoprotectants: the essential antifreezes to protect life in the frozen state. CryoLetters 25:375-388.
Gagliardi RF, Pacheco GP, Coculilo SP, Vall JFM, Mansur E (2000). *In vitro* plant regeneration from seed explants of wild groundnut species (Genus *Arachis*, Section *Extranervosae*). Biodiversity & Conservation 9:943-951.
Gagliardi RF, Pacheco GP, Vall JFM, Mansur E (2002). Cryopreservation of cultivated and wild Arachis species embryonic axes using desiccation and vitrification methods. CryoLetters 23 (1):61-68.
Helliot B, Panis B, Poumay Y, Swennen R, Lepoivre P, Frison E (2002).Cryopreservation for the elimination of cucumber mosaic and banana streak viruses from banana (*Musa* spp.). Plant Cell Reports. 20:1117-1122.
Jain SM (2011). Prospects of *in vitro* conservation of date palm genetic diversity for suitable production. Emir. J. Food Agric. 23:110-119.
Matsumoto T, Sakai A (2003). Cryopreservation of axillary shoot tips of in vitro-grown grape (*Vitis*) by a two-step vitrification protocol. Euphytica 131:299-304.
Matsumoto T, Sakai A, Yamada K (1994). Cryopreservation of in vitro-grown apical meristems of wasabi (*Wasabi japonica*) by vitrification and subsequent high plant regeneration. Plant Cell Reports 13:442-446.
Matsumoto T, Sakai A, Yamada K (1995). Cryopreservation of in vitro-grown apical meristems of lily (*Lilium japonicum*) by vitrification. Plant Cell Tissue Organ Culture 41:231-241.
Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.
Ozudogru EA, Ozden-Tokatli Y, Gumusel F, Benelli C, Lambardi M (2009). Development of a cryopreservation procedure for peanut (*Arachis hypogaea* L.) embryonic axes and its application to local Turkish germplasm. Adv. Hort. Sci. 23(1):41-48.
Roberts EH, Ellis RH (1989). Water and seed survival. Ann Bot.63:39-52.
SAS Institute Inc. (1990). SAS/STAT User's guide, version 6′ 4th edn. SAS Inc. Cary, NC, USA.
Shibli RA, Al-Juboory K (2000). Cryopreservation of 'Nabali' olive (*Olea europea* L.) somatic embryos by encapsulation-dehydration and encapsulation-vitrification. CryoLetters 21:357-366.
Takagi H (2000). Recent developments in cryopreservation of shoot apices of tropical species. In: Cryopreservation of Tropical Plant Germplasm. Current Research Progress and Application. JIRCAS International Agriculture series No. 8. IPGRI Rome, Italy. p.178-192.
Usman IS, Abdulmalik MM (2010). Cryopreservation of embryonic axes of maize (*Zea maize* L.) by vitrification protocol. Afr. J. Biotechnol. 9(52):8955-8957.
Vazquez -Yanes C, Arechiga MR (1996). Exsitu conservation of tropical rain forest seed; problems and perspectives. Interciencia 21:293-298.
Volk GM, Walters C (2006). Plant vitrification solution 2 lowers water content and alters freezing behavior in shoot tips during cryoprotection. Cryobiology 52(1):48-61.
Waliyar F, Kumar PL, Ntare BR, Monyo E, Nigam SN, Reddy AS, Osiru M, Diallo AT (2007). Groundnut Rosette Disease and its Management. ICRISAT Information Bulletin No.75 p. 2.
Yamuna G, Sumathi V, Geetha SP, Praveen K, Swapna N, Nirmal BK (2007). Cryopreservation of *in vitro* grown shoots of ginger (*Zingiber officinale* Rosc.). CryoLetters 28:241-25