

The Effect of Abscisic Acid and Sucrose on Post-Thaw Embryogenic Competence and Subsequent Plant Recovery from Embryogenic Calli of Cassava

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Abstract: The effect of sucrose with or without abscisic acid (ABA) in cryoprotective medium on post-thaw embryogenic competence and subsequent plant recovery of embryogenic callus clumps of cassava was investigated. Post-thaw viability depended on duration of cryoprotection on the 0.3M sucrose cryoprotective medium. The presence of only ABA in the cryoprotective medium resulted in callus proliferation but loss of post-thaw viability. However, the inclusion of sucrose with low concentrations of ABA (5-10 mg/l) in the cryoprotective medium decreased callus proliferation but subsequent cryopreservation resulted in post-thaw viability and/or embryogenic competence, except at the higher concentration of ABA (20mg/l) where there was complete loss of post-thaw viability. Also, callus clumps pre-treated with low concentrations of ABA with sucrose prior to cryopreservation resulted in significantly higher plant recovery than only sucrose pre-treated callus clumps. Plants recovered from cryopreserved callus clumps were phenotypically similar to non-cryopreserved callus clumps. These results indicate that cryopreservation of embryogenic calli of cassava is a feasible option for long-term conservation of the crops' genetic resources.

Key words: Abscisic acid . Cryopreservation . Embryogenic calli . Embryogenic competence

INTRODUCTION

Cassava (*Manihot esculenta* Crantz) is an important tropical tuber crop with a farmgate value of \$7,000 million [1]. Recent introductions to subsistence farmers of improved and high-yielding cultivars have yielded increased production of the crop, particularly in sub-Saharan Africa [2]. However, these improved and high yielding genotypes pose a genetic erosion and/or extinction threat to existing farmers' landraces, which harbour useful genes. Therefore, appropriate conservation methods need to be developed alongside breeding programmes to solve the problem of genetic erosion.

However, cassava is a vegetatively propagated crop as a result of high heterozygosity, poor seed set and germination [3]. Thus, seed storage is not a feasible option for long-term conservation of the crop. Also, the traditional *in vitro* storage via slow growth is limited by periodic subcultures often leading to somaclonal variation [4] and high labour cost. Cryopreservation of embryogenic calli could be useful for long-term conservation of cassava genetic resources and therefore alleviates the problem of tissue-culture-induced somaclonal variation.

Cryopreservation of embryogenic tissues of several crop plants including cassava has been reported [5-9].

In cassava, Mycock *et al.* [7] reported 40-60% post-thaw survival of heart-shaped or torpedo stage somatic embryos using vitrification-based method. Danso and Ford-Lloyd [9] reported 95% post-thaw viability with embryogenic calli containing proembryogenic masses or globular embryos after sucrose cryoprotection followed by air desiccation.

The inclusion of abscisic acid (ABA) to the sucrose enriched cryoprotective medium prior to cryopreservation has also been shown to influence post-thaw viability [10]. ABA improves embryogenic response in plant tissues [11] enhances maturation and desiccation tolerance of somatic embryos [12] and subsequent germination [13]. Consequently, many cryogenic protocols employ a preculture step of tissues on ABA prior to immersion in liquid nitrogen [14-16]. In this paper, the effect of sucrose with or without ABA on post-thaw viability and embryogenic competence of embryogenic tissues of cassava is reported. The effect of ABA on post cryopreservation plant recovery from embryogenic tissues of cassava is also reported.

MATERIALS AND METHODS

Plant materials and induction of somatic embryos: *In vitro* plantlets of cassava accession TME 9, TME 60444 and seed-derived genotypes of SM₁-2075-1

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Line 1 were used for this study because of their high embryogenetic response [16]. Plantlets of these accessions were maintained on solid Murashighe and Skoog [17] with vitamins (MS) medium supplemented with 0.05mg/l benzyl amino purine (BAP), 0.01mg/l naphthalene acetic acid (NAA). Primary and cyclic embryos from embryogenic axes of SM₁-2075-1 Line 1 as well as young leaf lobes from clonal accessions TME 9, Santom, M. Col 1505 and TME 60444 were produced as described [17]. All cultures were incubated at a temperature of 22-25°C, 16/8 h (light/dark) photoperiod and light intensity of 40 $\mu\text{mol m}^{-2}\text{sec}^{-1}$ in the growth room.

Effect of sucrose on cryopreservation of matured somatic embryos: Matured somatic embryos of TME 9 (21 d old) obtained from cyclic embryogenesis were transferred to 350 ml of liquid MS supplemented with 0.3M or 0.5M sucrose and placed on orbital shaker in the growth room. After 3 or 7 days the embryos were desiccated on Whatmann filter paper under the laminar flowhood to loose approximately 80% of their moisture. After desiccation, half of the embryos were cultured directly on MS medium supplemented with 0.1 mg/l BAP for germination (controls) while the remaining half were transferred to 1.8 ml cryovials and rapidly frozen in liquid nitrogen contained in a dewar flask for at least 20 minutes. The embryos were rapidly thawed in a warm water bath at 40°C for 90s. Thereafter, cryopreserved embryos were allowed to assume room temperature for 1 h and then transferred to sterile Whatman filter placed on 25 ml of solid MS medium supplemented with 0.3 M sucrose and incubated in the dark for 1 day. They were finally cultured for germination in the dark for further 7d prior to transfer to growth room culture conditions. After 14 days of culture, 30 embryos from each treatment including controls were put in Petri dishes and treated with 25 ml of 0.01% tetrazolium (TTZ) for 48 h to test for germination. The percentage of embryos with pink colour indicating germination competence was recorded.

Duration of induction, exposure to sucrose and viability: Proliferating embryogenic calli (300-350 mg) induced on 25ml of MS supplemented with 8 mg/l 2,4-D, 30 mg/l sucrose and 2 μM CuSO₄ for 10 d (induction medium, IM) were transferred to cryoprotective medium consisting of MS supplemented with 0.3M sucrose for 4, 10 or 21 d. Callus clumps were then desiccated for 16 h under laminar air flowhood. After desiccation, half of the embryogenic callus clumps were cryopreserved as described above while the other half served as controls. Both cryopreserved and

non-cryopreserved callus clumps were cultured on solid MS medium supplemented with 0.3 M sucrose and incubated in the dark for 1 d. Callus clumps were then transferred to 25 ml of solid MS medium supplemented with 0.1 mg/l BAP for 6 d in the dark prior to incubation in growth room conditions. Records on percentage viability as well as embryogenic competence were recorded 6 weeks after culture. The optimal protocol obtained in this experiment was applied to Santom, M. Col 1505, TME 60444 and seed derived genotype SM₁-2075-1 Line 1

Sucrose with or without ABA cryoprotection and post thaw viability: Embryogenic calli (350 mg) of TME 9 and SM₁-2075-1 Line 1 induced on IM were transferred to fresh IM supplemented with 0.3M sucrose plus 5, 10 or 20 mg/l ABA or IM supplemented with only 5, 10, 20 mg/l ABA. After 21 d, the weight of the calli was recorded and then desiccated for 14 h. Callus clumps were reweighed and the percentage moisture loss was calculated as the difference between the final and initial weight/final weight of clumps multiplied by 100. The desiccated clumps were then cryopreserved and cultured as described above. The percentage viability and/or embryogenic competence of the cryopreserved clumps were taken 4 weeks after culture.

ABA and post-thaw plant recovery: Matured somatic embryos obtained from cryopreserved clumps of TME 9 and SM₁-2075-1 Line 1, were fragmented and cultured on solid IM medium to generate cyclic embryos. These cyclic embryos were also recycled to generate more embryos in 350 ml of liquid MS medium supplemented with 10 mg/l NAA. About 90 to 120 matured cyclic embryos (24 d old) obtained from clumps cultured on media containing sucrose with or without ABA pre-treatments were isolated, desiccated in Petri dishes for 14 d and then cultured on solid MS medium supplemented with 0.1 mg/l BA and incubated at 37°C in an incubator. After 6 days, cultures were transfer to a growth room with a 16/8 h (light/dark) photoperiod and light intensity of 40 $\mu\text{mol m}^{-2}\text{s}^{-1}$. The number of embryos of embryos that developed shoots or roots was recorded. The plantlets obtained were examined for their phenotypic traits after eight weeks and were compared with non-cryopreserved plantlets.

Assessment for viability and statistical analysis: The viability of cryopreserved callus clumps was defined as the presence of proliferating callus, foliose structures, roots or somatic embryos, while embryogenicity refers to the number of viable clumps with cotyledonary-stage somatic embryos. Each experiment was replicated three

or four times with five embryogenic clumps per replicate unless otherwise stated. All statistical analyses were performed using MINITAB statistical package version 13.31

RESULTS

Response of matured somatic embryos to cryopreservation: Matured somatic embryos used for this work were obtained as previously reported (Danso and Ford-Lloyd, 2002; 2004). Both cryopreserved and non-cryopreserved (control) matured somatic embryos showed no signs of root or shoot development after 14 d of culture. Thus, embryos were tested for germination using the tetrazolium test (TTZ). After 24 h of TTZ treatment, the basal portion of the cotyledons as well as the hypocotyls turned pink; the intensity of the colour increased after 48 h of treatment indicating active germination activity. However, germination of the embryos as indicated by the pink colour was dependent on the sucrose concentration in the pre-treatment medium as well as the duration of the pre-treatment. Embryos precultured for 3 d prior to cryopreservation showed lower percentage of germination and were independent of the sucrose concentration in the pre-treatment medium (Table 1). Also, in both treatments the cryopreserved embryos showed higher percentage of germination activity than the non-cryopreserved controls. However, with embryos precultured for 7 d, the percentage germination of embryos pre-treated with 0.3M sucrose was significantly ($P<0.05$) higher than 0.5M sucrose (Table 1) indicating the detrimental effect of higher sucrose concentration on germination. Also, the non-cryopreserved controls were significantly higher than cryopreserved embryos precultured in 0.5M sucrose.

Response of callus clumps to duration of exposure to higher sucrose concentration: Following the loss of post-thaw viability by cryopreserved matured somatic embryos, embryogenic calli containing globular embryos were used as micropropagules for

cryopreservation. Ten days old desiccated embryogenic clumps without sucrose cryoprotection lost post-thaw viability while non-cryopreserved control callus clumps developed roots, somatic embryos or foliose structures (data not shown). In contrast, callus clumps cryoprotected on 0.3M sucrose for 4, 10 or 21 days followed by 16h desiccation and subsequent cryopreservation resulted in post-thaw viability and/or embryogenic competence indicating the importance of sucrose in cryoprotection (Fig. 1). The percentage post-thaw viability significantly ($P<0.05$) increased to 95% after 21 d of exposure compared to 25% and 55% after 4 or 10 d of exposure respectively. In addition, all the post-thaw viable callus clumps were embryogenically competent and there was no significant ($P<0.05$) difference between the cryopreserved and non-cryopreserved controls.

Application of optimal protocol to different genotypes: The optimal protocol of induction of embryogenic calli on IM for 10 days, cryoprotection on 0.3M sucrose for 21 d followed by 16h of desiccation prior to rapid immersion in liquid nitrogen was applied to Santom, M.Col 1505, TME 60444 and seed-derived genotype SM₁-2075-1 Line 1 using TME 9 as control. Results on post-thaw viability and subsequent embryogenic competence are shown in Fig. 2. Post-thaw viability of callus clumps varied depending on the accession and ranged from 5% in M. Col 1505 to 95% in TME 9. Consequently, subsequent embryogenic competence also varied. In TME 9 and SM₁-2075-1 Line 1, post-thaw embryogenic competence was higher than 60% while in the remaining genotypes embryogenic competence was below 40%. With the exception of TME 60444 and Santom, the embryogenic competence of cryopreserved callus clumps was comparatively higher than non-cryopreserved controls. Of all the genotypes tested, M. Col 1505 showed the lowest response causing highly significant differences ($P<0.05$) in post-thaw viability and subsequent embryogenicity between the accessions. However, there were no significant differences between mean numbers of embryos produced per clump.

Table 1: Effect of sucrose on percentage germination (tested by the TTZ test) of matured somatic embryos after cryopreservation.

Sucrose concentration (M)	Germination (%) Duration of sucrose preculture (days)			
	3 d		7d	
	Control	Cryopreserved	Control	Cryopreserved
0.3	36.0aA	43.3aA	100aA	83.0aA
0.5	26.6aA	30.0aA	100aA	53.3bB

Figure in a column indicated by the same lower case letters are not significantly different at ($P<0.05$) according to Tukey's pairwise comparisons. Similarly, figures in a row indicated by the same upper case letters are not significantly different at ($P<0.05$)

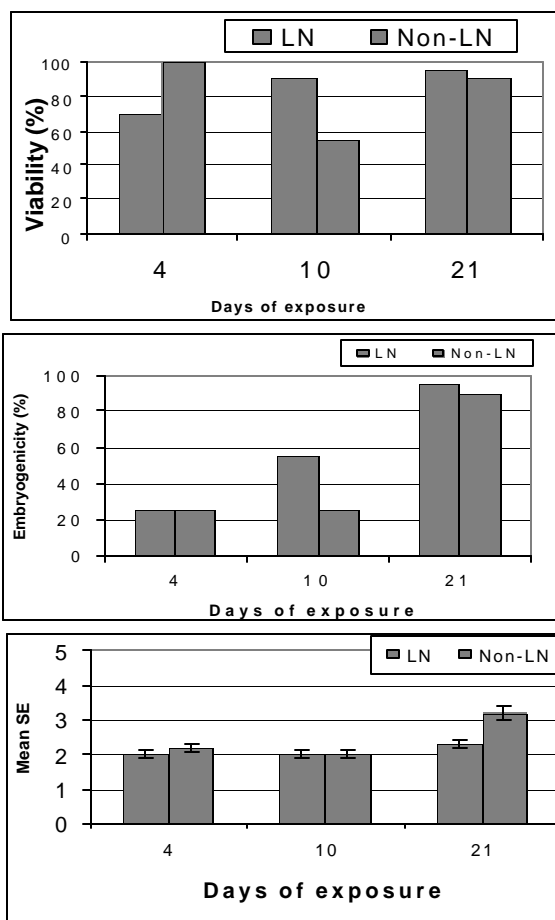


Fig. 1: Effect of duration of exposure on 0.3 M sucrose on post-thaw viability and embryogenic competence of cryopreserved embryogenic clumps of TME 9. Embryogenic clumps were induced without sucrose followed by cryoprotection on 0.3M sucrose for 4, 10 or 21 days and 16 hours of dehydration. Each treatment was replicated 4 times with 5 embryogenic clumps per treatment. Bars indicate standard error

Table 2: Effect of ABA in cryoprotective medium with or without sucrose on growth of callus clumps after 21 days of culture

Treatment	Weight gain or loss after 21 d/mg	
	With 0.3M sucrose	Without sucrose
5 mg/l ABA	0.047±0.01a	13.87±0.05b
10 mg/l ABA	0.564±0.1a	13.96±1.3b
20 mg/l ABA	-0.053±0.2ab	13.07±0.6b

Figures in a row followed by the same letters are not significant different at (P<0.05) according to Tukey's pairwise comparisons. Also, figures in a column followed by the same letters are not significantly different. Values represent 15-20 embryogenic clumps

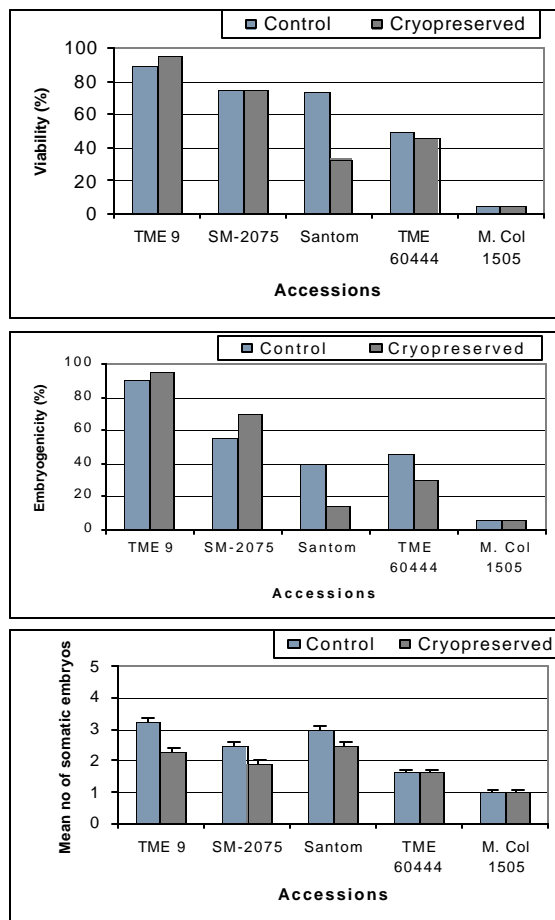


Fig. 2: Post-thaw survival of callus clumps of 5 cassava accessions 4 weeks after culture. Callus clumps were induced on induction media (IM) with 30 mg/l sucrose for 10 days and then transferred to 0.3M sucrose for 21 days prior to cryopreservation

Effect of sucrose with ABA on post-thaw embryogenic competence: To enhance post-thaw embryogenic competence, the effect of 5, 10 and 20 mg/l ABA with or without 0.3 M sucrose was tested on accession TME 9 and thereafter the optimal ABA concentration was applied to embryogenic calli of seed-derived genotype SM₁-2075-1 Line 1. The presence of only ABA in the cryoprotective medium did not achieve the expected effect of dehydration during preculture; rather there was profuse growth of callus, which resulted in weight gain 21 d after culture independent of the concentration of ABA in the cryoprotective medium (Table 2). In contrast, the presence of sucrose with ABA significantly (P<0.05) decreased callus growth compared to only ABA treatment and at the highest concentration of 20 mg/l ABA plus sucrose there was dehydration of callus clumps (Table 2)

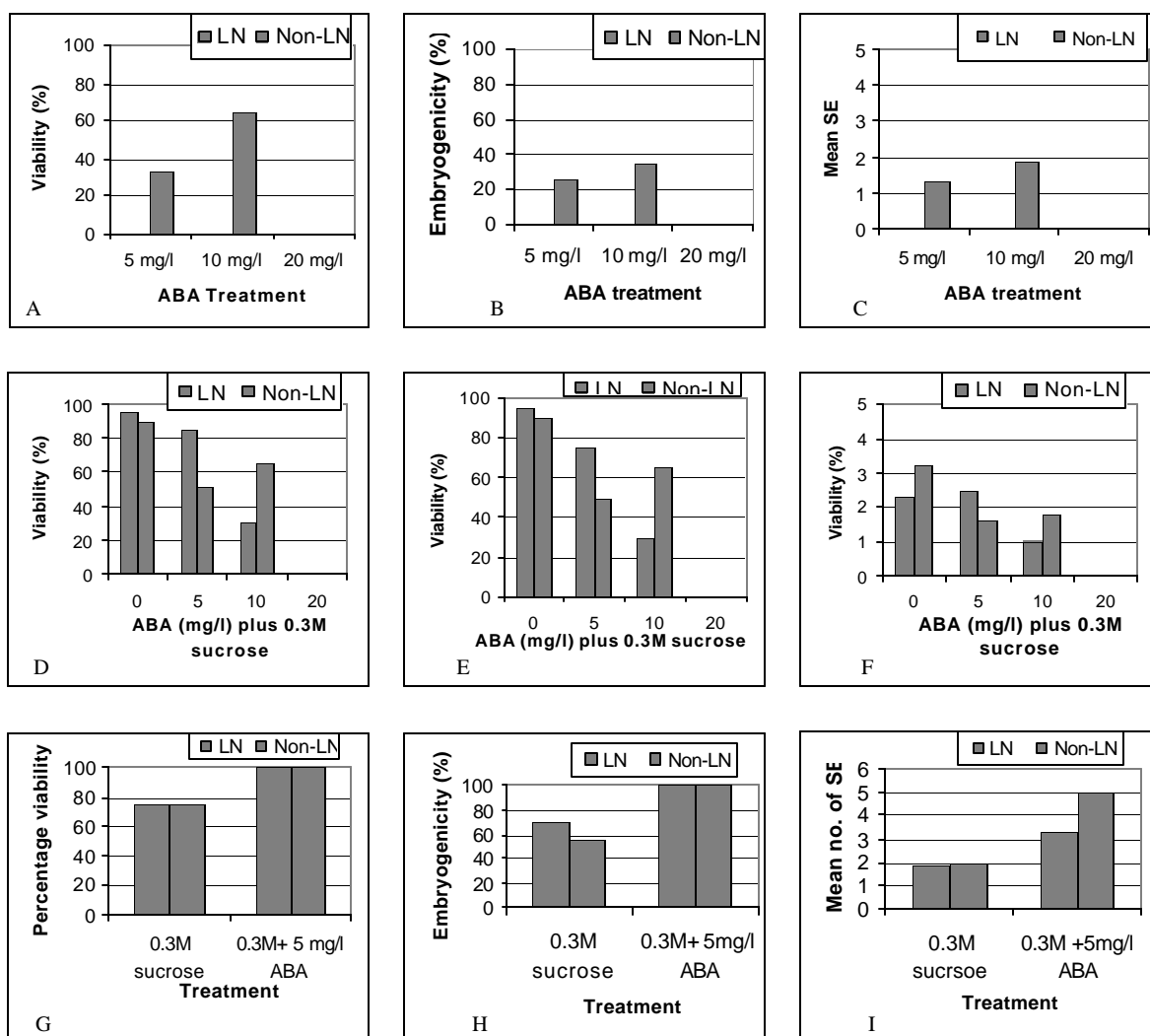


Fig. 3: Effect of ABA with or without sucrose on post-thaw embryogenic competence of embryogenic calli of cassava accession TME 9 (a-f) and SM1-2075-1 Line 1 (g-j). Clumps were induced on 0.0 M sucrose and thereafter transferred to 0.3M sucrose for 21 days prior to 14 hours of dehydration and cryopreservation

For subsequent cryopreservation, callus clumps treated with sucrose with or without ABA were desiccated to loose 80-82% of their moisture as previously reported (9). Cryopreservation of callus clumps cryoprotected with 5-20 mg/l ABA without sucrose lost post thaw viability (Fig. 3a-c). Although non-cryopreserved controls were embryogenically competent, 20 mg/l ABA had a detrimental effect on somatic embryo production. Post-thaw viability and/or embryogenic competence of callus clumps was achieved with 5 or 10 mg/l ABA only when there was 0.3M sucrose in the cryoprotective medium (Fig. 3d-f). Two-way analysis of variance showed that post-thaw viability and embryogenicity of cryopreserved callus clumps of accession TME 9 significantly ($P < 0.05$) decreased with increased concentration of ABA. At a

concentration of 20 mg/l, ABA had a detrimental effect on post-thaw viability and subsequent embryogenicity. In the presence of 5 mg/l ABA, embryogenic competence and mean number of embryos per callus of cryopreserved callus clumps was comparatively higher than non-cryopreserved controls but this increase is not significantly different at $P < 0.05$ (Fig. 4d-f). When this optimal ABA with sucrose pre-treatment was applied to accession SM₁-2075-1 Line 1, post-thaw embryogenic competence was 100% compared to 70% for callus clumps pretreated with only sucrose treatment (Fig. 3g-j). There was no significant difference between embryogenicity of callus clumps treated with sucrose and those of sucrose plus ABA. However, the mean number of somatic embryos produced was significantly different ($P < 0.05$).

Table 3: Post-thaw plant recovery from cryopreserved somatic embryos of cassava accessions TME 9 and SM₁-2075-1 Line 1. Somatic embryos were desiccated in Petri dishes for 14 d after which they lost 70-75% of their moisture and then cultured on MS medium supplemented with 0.1 mg/l BAP

Accession	Treatment ^a	Shoot or root formation (%)		
		Normal shoots	Malformed shoots	Roots
TME 9	Control	30.0a	30.0a	10.0a
	Sucrose only	20.0aA	32.5b	0.0b
	Sucrose plus ABA	70.0b	3.3c	53.3c
SM1-2075-1 Line1	Control	16.7a	63.3a	20.0a
	Sucrose	55.0bB	17.5b	77.5b
	Sucrose plus ABA	58.3b	27.1b	85.4b

Figures in a column followed by the same letters are not significantly different according to ?² pairwise comparisons. Upper case letters indicate significant differences in the germination response of cryopreserved somatic embryos of TME 9 and SM1-2075-Line 1 treated with only sucrose. Number of embryos cultured varied from 40 to 60 per treatment

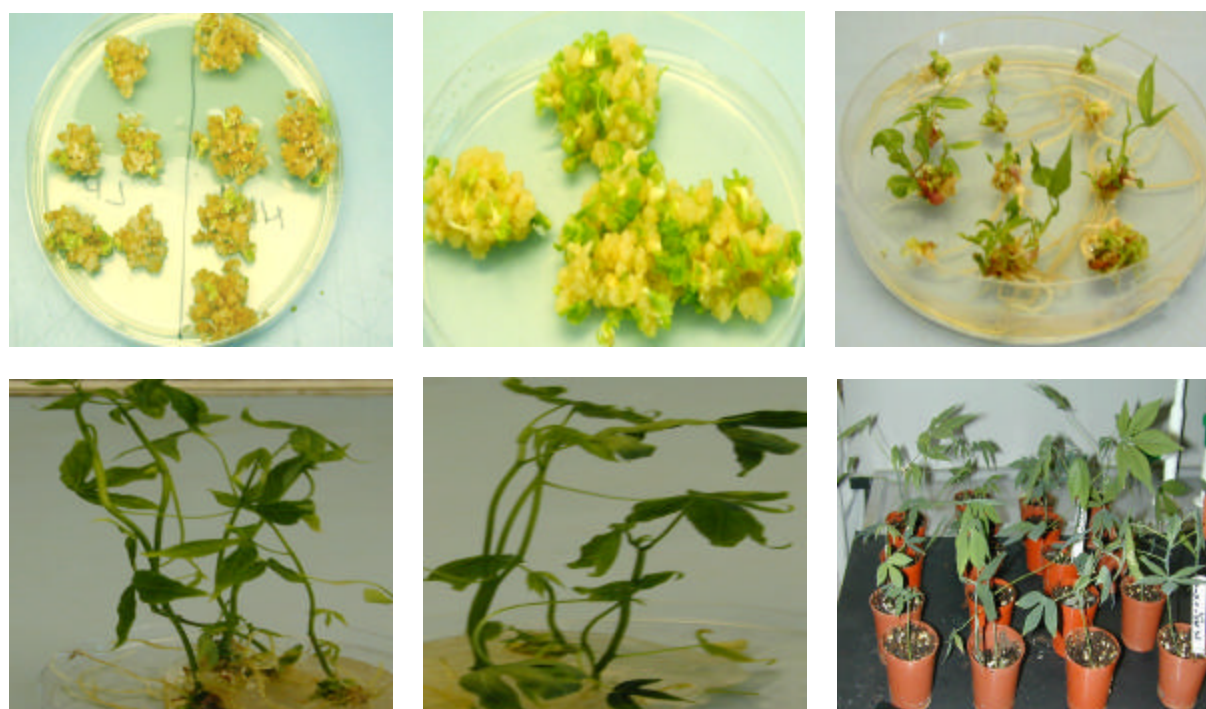


Fig. 4: Development of somatic embryos from cryopreserved embryogenic dumps of cassava. A: cryopreserved embryogenic clumps developing somatic embryos; B Cryopreserved somatic embryos undergoing cyclic embryogenesis and C: plantlets developing from matured desiccated cryopreserved somatic embryos of Santom D. Fully developed plantlets derived from ABA treated non-cryopreserved and (E) cryopreserved somatic embryos of cassava accession SM-2075-1 Line 1 five weeks after culture; F Cryopreserved plants of SM-2075-1 Line 1 growing in pots. Bars = 10mm

Post cryopreservation plant recovery: Somatic embryos obtained from cryopreserved embryogenic clumps of genotypes were used to establish post-thaw cyclic embryogenesis and subsequent plant regeneration (Fig. 4). However, only TME 9 and SM₁-2075-1 Line 1 were used to study the effect of

ABA treatment on plant recovery. Somatic embryos from cryopreserved derived callus clumps were morphologically similar to non-cryopreserved embryos. When mature cryopreserved embryos from ABA or non-ABA pre-treated callus clumps were desiccated and cultured on MS medium supplemented with

0.1 mg/l BAP and incubated at 37°C there was plant recovery (Table 3, Fig. 4C and D). Embryos developed roots within 6 d followed by emergence of shoots. Plants obtained had well developed shoots with or without roots and were phenotypically similar to the non-cryopreserved controls. There were significant differences in the response of cryopreserved somatic embryos to germination (Table 3). Among the three accessions tested for plant recovery, TME 9 had lowest percentage (20%) of plant recovery when embryos were treated with only sucrose (Table 3).

The percentage of plantlets derived from cryopreserved somatic embryos treated with ABA was significantly ($P < 0.05$) higher than the controls indicating that cryopreservation improve morphogenesis (Table 3). In accession TME 9, cryopreservation with ABA significantly increased plant development than without ABA.

DISCUSSION

Abscisic acid together with sucrose is known to influence stress physiology of plants and thus have been employed in cryogenic procedures for long-term conservation of plant genetic resources [6, 10]. Thus, we studied the effect of sucrose with or without ABA on cryopreservation of matured somatic embryos and embryogenic tissues of cassava and their subsequent plant recovery.

Both cryopreserved and non-cryopreserved whole matured somatic embryos pretreated with sucrose neither develop neither shoots nor roots. This observation has also been reported in *Quercus robur* using desiccation and vitrification cryogenic procedures [6]. Thus, TTZ was used to test for germination activities in both cryopreserved and non-cryopreserved matured somatic embryos. The TTZ test showed active sign of germination in all treatments (indicated by pink coloration). However, embryos precultured in 0.3 M sucrose for 7 d showed higher percentage of germination activity than those precultured in 0.5 M suggesting that prolong cryoprotection in sucrose enhances post-thaw germination. Although TTZ test is subjective, it has been used to test for post-thaw germination in cryogenic studies [7]. The inability of the whole cryopreserved somatic embryos to germinate could be attributed to either the presence of high sucrose concentration in the pre-treatment medium or the fast desiccation method applied prior to cryopreservation. It has been shown that fast desiccation reduce germination in black spruce somatic embryos [18] while slow desiccation enhances germination.

The presence of high concentration of ABA (20 mg/l) with sucrose achieved osmotic dehydration of callus clumps during cryoprotection while low concentrations (5 and 10 mg/l) with or without ABA stimulated callus growth. Although ABA and sucrose is usually inhibitory to callus growth low concentrations are known to stimulate its growth. In haploid tobacco callus, 0.1 mg/l ABA stimulated callus growth but was inhibitory at 10 mg/l [19]. Leon and Sheen [20] have reported that glucose and abscisic acid promote growth at low concentrations but act synergistically to inhibit growth at high concentrations. This observation is similar to the results presented in this study where low concentration of ABA with sucrose in the cryoprotective medium enhanced callus growth.

On subsequent cryopreservation, the presence of 5 mg/l ABA with sucrose resulted in comparatively higher post thaw embryogenic competence in SM₁-2075-1 Line 1 and mean number of embryos per callus in TME 9 while callus clumps treated with only ABA lost post thaw viability. ABA is known to play a central role in adaptive responses to environmental cues such as drought and freezing [20], enhance formation germination [13] and to avoid precocious germination [21]. Evidence suggests that application of exogenous ABA enhanced dehydration and /or freezing tolerance of cell cultures through ABA-responsive genes and ABA-responsive proteins [12, 20] have reported that ABA induced accumulation of sugars, heat-stable proteins and dehydrins in protocorm-like bodies of *Dendrobium candidum*. The comparatively higher post-thaw embryogenic competence achieved in callus clumps of accession SM₁-2075-1 Line 1 in the presence of ABA with sucrose may therefore be due to the dual role of ABA in increasing frequency of embryo maturation and induction of freezing tolerance.

Although higher levels of 20 mg/l ABA with sucrose in the cryoprotective medium achieved osmotic dehydration prior to desiccation and cryopreservation, it was inhibitory to post-thaw embryogenic competence indicating the inhibitory effect of high levels of ABA. This observation compares with that of [22] who reported that 11-21 mg/l ABA suppressed embryo formation in *Citrus sinensis*. In this study, the inhibition of post-thaw viability may be due to the synergistic effect of sucrose and ABA in the cryoprotective medium.

Somatic embryos derived from both sucrose with or without ABA treated cryopreserved callus clumps developed cyclic embryos; desiccation and culture led to successful plant recovery. The percentage of normal shoots obtained from cryopreserved embryos with ABA treatment was significantly higher than

non-cryopreserved embryos and those treated with sucrose only; thus cryopreservation enhanced the morphogenic potential of cassava somatic embryos. According to [23] cryopreservation is used to circumvent the loss of embryogenic and organogenic competence as well as loss of vigour of *in vitro* cultures. The high frequency of germination in ABA treated somatic embryo derived plants could be attributed to the growth regulator playing an active role in maturation and germination of the embryos. Although ABA is considered as a stress hormone and growth inhibitor, recent evidence suggests that it plays an active role in root and shoot growth [24]. In cassava, it has been shown that pre-treatment of somatic embryos with 0.1 $\mu\text{mol/l}$ ABA prior to desiccation significantly enhanced germination [13].

The cryogenic procedure developed in this study has shown that post-thaw embryogenic competence as well as plant recovery is enhanced by preculture of embryogenic tissues on a medium containing ABA. This simplified cryogenic procedure could be used to complement 'on farm' conservation of diverse cassava germplasm in farmer's fields as well as other *in vitro* methods of conservation of the crop.

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