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A methodology for recovering cassava plants from shoot tips maintained in liquid nitrogen

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Abstract Shoot tips of in vitro-grown plantlets of cassava (*Manihot esculenta* Crantz), representing a wide range of germplasm, were cryopreserved as follows: precultured for 3 days, cryoprotected and dehydrated for 1 h, then frozen in liquid nitrogen using a six-step protocol. After 3 h in liquid nitrogen, the shoot tips were removed, rapidly warmed, and recultured sequentially in three recovery media. After 2 weeks, the regeneration of frozen shoot tips was completed. Genotypes with a low response were identified. Their response was attributed to the effects of pre- and post-freezing steps. Refining the methodology led to a consistent 50–70% plant recovery.

Key words Cassava (*Manihot esculenta* Crantz) · Cryopreservation

Abbreviations *DMSO* Dimethylsulfoxide · *MS* Murashige and Skoog medium (1962) · *LN* liquid nitrogen

Introduction

Cassava (*Manihot esculenta* Crantz) is the main source of dietary calories for more than 500 million people in the tropics. Vegetative propagation, bulkiness of planting material, and risk of genetic erosion make cassava an ideal candidate for the application of innovative germplasm conservation. The conventional method of cassava germplasm conservation is continuous clonal cultivation in the field. In field maintenance, however, valuable materials are often exposed to pests, diseases, and soil or climatic stresses. An in vitro gene bank has been developed at CIAT in which nearly 6,000 cassava clones from 23 countries are maintained under slow growth (Roca et al. 1989). The in vitro gene bank at CIAT constitutes an active collection, in which clones are recycled every 12–18 months.

Cryopreservation would permit the long-term storage of cassava in a reduced space, free of genetic change, and at low cost. Whereas we have been unable to reproduce Bajaj's results (1977), Kartha et al. (1982) provided useful information on pre-culture conditions and the cryoprotection of cassava meristems. The research reported here, conducted at CIAT, was aimed at developing a protocol for recovering cassava plants from shoot tips stored in liquid nitrogen (LN). The main components of the protocol are pre-culture, cryoprotection and dehydration treatments, freezing of shoot tip explants, storage in LN, and the use of recovery media to allow the growth of complete plants.

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Materials and methods

Plant material

Shoot tips of cassava (*Manihot esculenta* Cranz) comprising the apical dome and two to three of the youngest leaf primordia, were used throughout, unless stated otherwise. The shoot-tip explants were obtained from plantlets grown in vitro. These plantlets had been generated, using apical meristems, from sprouts grown on stem cuttings in the greenhouse and cultured in 4E medium (Roca 1984). After 4–5 weeks the terminal bud and any visible axillary buds growing from the apical meristems were excised and cultured in Magenta G-7 vessels containing 4E medium and under culture conditions of 26–28°C, a light intensity of 15 $\mu Em^{-2}s^{-1}$, and a 12-h (day); 12-h (night) photoperiod. After 3–4 weeks, the plantlets, 4–6 cm tall, were ready for use as a source of shoot tips for the cryopreservation experiments. Cassava cv 'MCol 22' was used throughout most of this study and 14 other cultivars were added to study genotypic effects.

Osmotic agents and abscisic acid (ABA)

Using the 4E medium as a base, we applied six different treatments to determine the effect of some osmotic agents and ABA on tissue viability and shoot formation at the end of pre-culture and without freezing. The 4E medium is a modification of the Murashige and

Skoog medium (1962) developed by Roca (1984) and contains 1.7×10^{-7} *M* 6-benzylamino purine (BAP), 1.44×10^{-7} *M* gibberellic acid (GA), 1.07×10^{-7} *M* 1-naphthaleneacetic acid (NAA), 2.96×10^{-6} *M* thiamine-HCl, 5.55×10^{-4} *M* inositol, and 0.7% agar, pH 5.7–5.8. When ABA was used in the medium, BAP was omitted. The treatments were:

1) 4E medium, supplemented with 0.25, 0.5, 0.75, and 1 *M* sorbitol over a period of 7, 10, 15, and 20 days each
2) 4E medium, supplemented with 1.5, 2.5, 3.5, and 5 *M* sorbitol for 1, 3, 5, and 7 days each
3) 4E medium, supplemented with 1 *M* sorbitol and 0.25, 0.5, 0.75, and 1 *M* sucrose for 1, 3, 5, and 7 days each
4) 4E medium, supplemented with 1 *M* sorbitol and 0.001, 0.01, 0.1, and 1 *M* dimethylsulfoxide (DMSO) for 1, 3, 5, and 7 days each
5) 4E medium supplemented with 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-10} *M* ABA for 1, 3, 5, and 7 days each
6) 4E medium, supplemented with 1 *M* sorbitol and 10^{-3}, 10^{-5}, 10^{-7}, and 10^{-10} *M* ABA for 1, 3, 5, and 7 days each

A total of 2 600 shoot tips of cv 'MCol 22' were used for this experiment. Of these, 200 explants were kept throughout the trials in 4E medium without supplements as control. Each treatment consisted of 400 shoot tips, thus 25 shoot tips were used per each individual sub-treatment. Cultures were maintained at 26°–28°C, a light intensity of 15 $\mu Em^{-2}s^{-1}$and a 12 h/day photoperiod. Explants were placed in petri dishes since this experiment did not involve freezing. At the end of each treatment, 15 shoot tips were taken at random and transferred to 4E medium for regrowth. Two parameters were measured: viability (survival of tissues growing in any form; e.g., callus with or without shoot primordia) and shoot growth (regrown tissue with plantlet recovery) after 4 weeks.

Shoot-tip size

Shoot-tip explants were either small (1–2 mm in height) or large (3–4 mm in height). A total of 110 explants were used per treatment. Shoot-tips were precultured in C4 medium (MS salts with 1 *M* sorbitol, 0.1 *M* DMSO, and 4% sucrose) at 26°–28°C in the dark, before cryoprotection. Cryoprotection was carried out in 10% DMSO and 1 *M* sorbitol for 2 h on ice. After cryoprotection, the cryoprotectants were removed and the shoot tips were dehydrated for 1 h on Whatman No. 2 filter paper placed inside the petri dishes. Shoot tips were dried at room temperature (24°–26°C) and on ice; 10 shoot tips were used per temperature treatment and transferred to ampoules for freezing. The freezing protocol used in this experiment was 0.5°C per minute to –15°C, with rapid changes between –15°C and –17°C, then 1°C per minute to –40°C. After freezing to –40°C, the explants were immersed directly in LN for 3 h. Frozen explants were thawed at 37°C and then recovered sequentially in two media for 2 days each, R1 (0.75 *M* sucrose with 0.2% activated charcoal) and R2 (half-strength MS with 0.35 *M* sucrose and 5.56×10^{-3} *M* inositol), at 25°C in the dark. The explants were then transferred to a third semisolid medium (4E) at 28°C and under a light intensity of 15 $\mu Em^{-2}s^{-1}$.

Genotypic response

Fourteen cassava varieties from the in vitro collection maintained at CIAT were randomly selected for use in this experiment: 'MArg 2', 'MBra 161', 'MCol 1468', 'MCR 113', 'MCub 27', 'MDom 2', 'MEcu 48', 'MGua 14', 'MMex 71', 'MPan 125', 'MPar 193', 'MPer 303', 'MVen 232', and 'CM 922–2'. 'MCol 22' was used as control. The number of shoot tips per cassava variety varied from 86 to 120.

The cryopreservation protocol applied to material in this part of the work included the best conditions with respect to explant size, pre-culture medium, tissue dehydration, and recovery media, which were obtained fom the earlier parts of this study.

1) Explant: shoot tips (2 mm in height);
2) Pre-culture: C4 medium for 3 days in the dark at 26°–28°C;
3) Cryoprotection with: 1 *M* sorbitol, 10% DMSO, and 4% sucrose for 2 h on ice;
4) Tissue dehydration: on filter paper for 1 h;
5) Freezing with a Cryomed 1010 machine: (1) 5°C wait chamber, (2) 0.5°C per minute until –15°C in sample, (3) 25°C to –20°C in chamber, (4) 15°C to –17°C in chamber, (5) 1°C per minute to –40°C in sample;
6) Immersion and storage in liquid nitrogen;
7) Thawing at 37°C;
8) Recovery: R1 medium followed by R2 medium for 2 days each, then transferred to semisolid 4E medium; and
9) Evaluation: tissue viability and shoot growth after 1 month.

Ten explants from each variety were placed on 4E medium in petri dishes and kept, as control under the conditions described above.

Results and discussion

Effect of osmotic agents and ABA

This part of the work was aimed at observing the effect of media components on tissue regrowth at the end of preculture, without freezing. Table 1 shows the overall effect of treatments on tissue viability and shoot growth, and Table 2 compares the effects of individual sub-treatments on shoot growth over time. A nested statistical analysis was used to assess the overall effect of the six treatments, each one including four defined concentrations of medium constituents, supplied over four time periods, as described in the Materials and methods. Significant differences between treatments were analyzed with Duncan's Multiple Range Test for means separation.

The six treatments resulted in statistically significant overall effects on tissue viability and shoot growth (Table 1). Shoot-tips responded best to sorbitol at low concentrations and ABA (treatments 1 and 5, Table 1). When sorbitol concentrations were too low (i.e., 0.25 and 0.5 *M*) viability and shoot growth were not significantly affected, whereas higher concentrations (i.e., 0.75 and 1 *M*) reduced both parameters (Table 2). The addition of sorbitol at 1 *M* increased tissue viability and shoot growth better when used in combination with ABA than with sucrose (treatments 3 and 6, Tables 1 and 2). Sorbitol concentrations higher than 1 *M* were detrimental to shoot growth, suggesting that 1 *M* sorbitol may be the maximum tolerated by cassava shoot-tips. Under these conditions, callusing at the base of the explants occurred, which, in turn, retarded shoot growth. Higher concentrations of sorbitol (3.5 *M* and 5 *M*) drastically inhibited not only callus formation but also shoot growth (see treatment 2 in Table 1 and sub-treatment 2.2, 2.4 in Table 2). With the exception of 0.25 *M* sucrose, all of the sucrose concentrations in combination with 1 *M* sorbitol, had a negative effect on tissue viability (treatment 3, Table 2). DMSO at low concentrations was less detrimental to tissue viability and shoot growth when used in combination with 1 *M* sorbitol (treatment 4, Table 2). Kartha et al. (1988) demonstrated that sorbitol could reverse the toxic effects caused by DMSO. The study here shows that the highest concentration of DMSO used (1 *M*) in combination with 1 *M* sorbitol (treatment 4.4 in Table 2) was toxic to cassava shoot tips. Most cryoprotectants

Table 1 Overall effect of pre-culture media containing sorbitol, DMSO and ABA on the viability and growth of cassava (cv 'MCol 22') shoot-tips without freezing

Treatment[b]	Average value (%)[a]	
	Viability	Shoot growth
1. Low sorbitol	95.25 b	81.16 b
2. High sorbitol	9.25 f	14.16 f
3. Sorbitol + sucrose	41.31 e	44.16 e
4. Sorbitol + DMSO	82.0 d	72.88 c
5. ABA	100 a	89.75 a
6. Sorbitol + ABA	88.25 c	59.55 d

[a] Average values with the same letter and in the same column are not significantly different at 0.05 level using Duncan's Multiple Range Test
[b] Nested analysis allows each treatment to be evaluated as a whole, with attention paid to the type of constituent and not to the concentrations or duration of treatment

Table 2 Effect of individual pre-culture sub-treatment containing sorbitol, DMSO, sucrose and ABA on shoot growth of cassava (cv 'MCol 22') shoot-tips without freezing

Treatment[a]	Sub-treatment	Shoot growth (%) Duration[b]			
		a	b	c	d
1	1.1 0.25 *M* sorbitol	96	100	100	96
	1.2 0.5 *M* sorbitol	100	100	100	96
	1.3 0.75 *M* sorbitol	76	52	84	48
	1.4 1 *M* sorbitol	64	52	48	32
2	2.1 1.5 *M* sorbitol	16	0	0	0
	2.2 2.5 *M* sorbitol	0	0	0	0
	2.3 3.5 *M* sorbitol	0	0	0	0
	2.4 5 *M* sorbitol	0	0	0	0
3	3.1 1.0 *M* sorbitol + 0.25 *M* sucrose	52	56	64	76
	3.2 1.0 *M* sorbitol + 0.5 *M* sucrose	32	0	0	0
	3.3 1.0 *M* sorbitol + 0.75 *M* sucrose	24	0	0	0
	3.4 1.0 *M* sorbitol + 1 *M* sucrose	32	4	0	0
4	4.1 1.0 *M* sorbitol + 0.001 *M* DMSO	68	76	92	92
	4.2 1.0 *M* sorbitol + 0.01 *M* DMSO	84	84	80	76
	4.3 1.0 *M* sorbitol + 0.1 *M* DMSO	68	80	96	76
	4.4 1.0 *M* sorbitol + 1 *M* DMSO	8	16	0	0
5	5.1 10^{-3} *M* ABA	96	100	0	60
	5.2 10^{-5} *M* ABA	100	100	88	96
	5.3 10^{-7} *M* ABA	100	100	96	100
	5.4 10^{-10} *M* ABA	100	100	100	100
6	6.1 1.0 *M* sorbitol + 10^{-3} *M* ABA	56	56	8	0
	6.2 1.0 *M* sorbitol + 10^{-5} *M* ABA	56	96	56	8
	6.3 1.0 *M* sorbitol + 10^{-7} *M* ABA	44	92	84	24
	6.4 1.0 *M* sorbitol + 10^{-10} *M* ABA	64	100	83	56

[a] For treatment description see Materials and methods and Table 1
[b] For sub-treatment durations see Materials and methods

are known to exhibit varying degrees of cytotoxicity at a high concentration. DMSO also penetrates the cell quickly and can act as a toxin; its toxicity is probably a result of both osmotic and biochemical effects (Benson 1990).

Table 3 Effect of shoot-tip size and tissue dehydration under two temperatures on viability and growth after freezing cassava (cv 'MCol 22') shoot-tips in liquid nitrogen

Shoot-tip size (height)	Dehydration temperature	Average value (%)[a]	
		Viability	Shoot growth
Small (1–2 mm)	At 26°–28°C	79.5 a	64 a
	On ice	63.1 b	31.6 b
Large (3–4 mm)	At 26°–28°C	2 a	0 b
	On ice	8 b	0 b

[a] Average values with the same letter and in the same column are not significantly different at the 0.05 level using Duncan's Multiple Range Test

Table 4 Response of 15 cassava genotypes to cryopreservation protocol

Cassava variety	Tissue culture response (%)	Average value (%)[a]	
		Viability	Shoot growth
M Col 22	100	92.72 a	52.22 a
CM 922-2	100	81.39 ab	48.65 a
M Arg 2	100	67.72 b	37.45 a
M Col 1468	100	73.73 b	10.00 bc
M Ecu 48	100	32.94 de	21.03 b
M Par 193	100	51.66 c	13.97 bc
M Pan 125	100	24.83 ef	12.92 bc
M Cub 27	100	45.31 dc	11.42 bc
M Dom 2	100	5.25 gh	3.12 c
M CR 113	100	14.4 fgh	2.77 c
M Gua 14	100	1.22 efg	2.00 c
M Per 303	100	15.26 fgh	0 c
M Bra 161	100	11 fgh	0 c
M Ven 232	100	5.02 gh	0 c
M Mex 71	100	3.9 h	0 c

[a] Average values with the same letter are not significantly different at the 0.05 level using Duncan's Multiple Range Test

When the pre-culture medium contained ABA at the lowest concentration tested (10^{-10} *M*) tissue viability and shoot growth was highest among all treatments without freezing (Tables 1 and 2). However, ABA was not included in our final protocol because the cryoprotection obtained at –20°C, when ABA alone or in combination with sorbitol was present in the pre-culture medium, was not higher than 40% in terms of shoot growth, and after freezing in LN, the recovery of plants was always arrested (data not shown). Here, the combination of 1 *M* sorbitol and 0.1 *M* DMSO proved to be the best combination when tissue was frozen in LN. Experiments carried out recently (data not shown) have shown that the addition of sucrose to the pre-culture medium at concentrations of 0.058 *M*–0.117 *M* considerably improves the response of cassava shoot-tips after freezing. On examining the pre-culture media used with freezing, we found the best contained supplements of 1 *M* sorbitol, 0.1 *M* DMSO, and 0.117 *M* (4%) sucrose. This medium has been named C4.

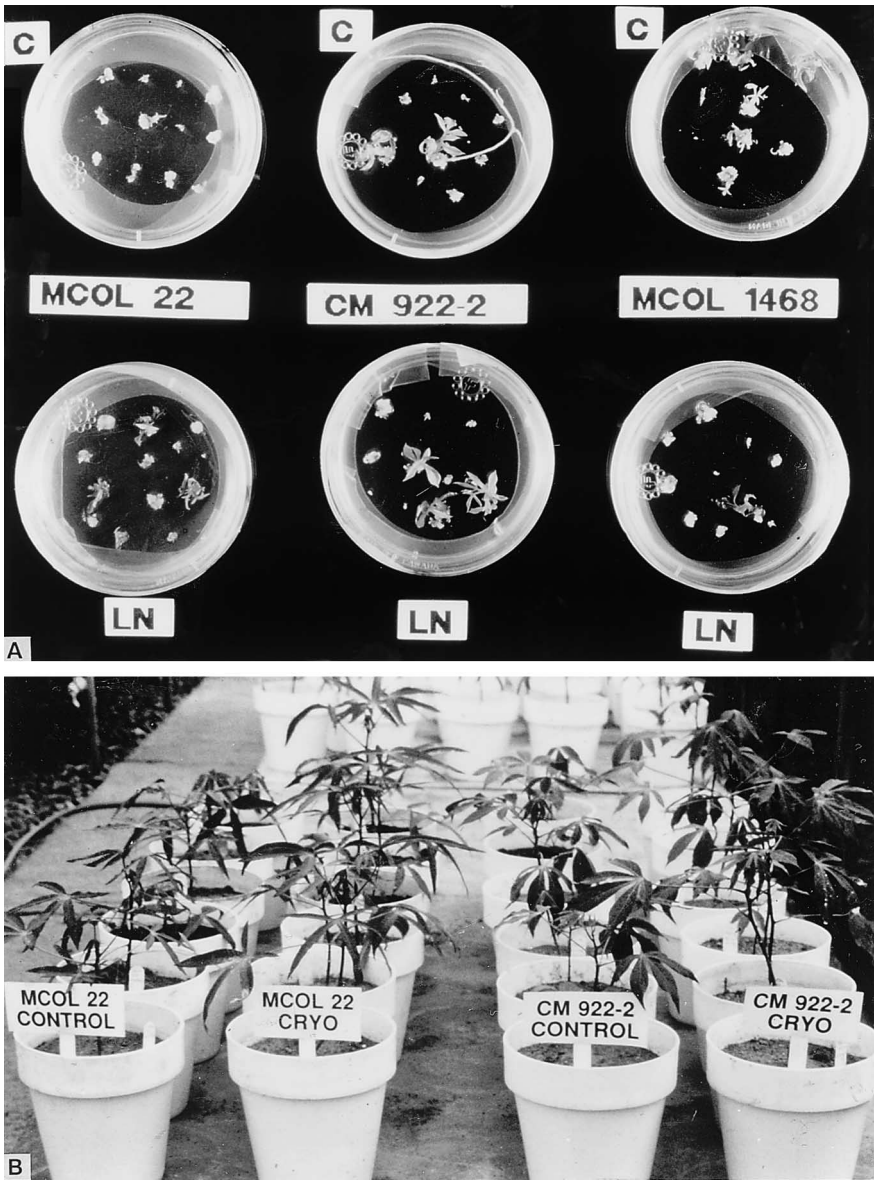

Fig. 1A, B Recovery of viable cassava shoots and plants from cryopreserved shoot-tips in liquid nitrogen. **A** Initiation of shoot growth from cryopreserved shoot-tips (*lower row*) and comparison with control shoot-tips, cryoprotected, but without freezing (*upper row*), of 3 cassava varieties. **B** Two-month-old cassava plants obtained from cryopreserved and control shoot-tips. Complete plants are produced by rooting viable shoots on 17 N medium (Roca 1984). After 3–4 weeks rooted plantlets are potted and gradually exposed to mesh-house conditions

Effect of shoot-tip size

Viability and shoot growth increased significantly when small shoot-tips were used as explants for cryopreservation (Table 3). The dehydration of shoot-tips before freezing was found to significantly improve tissue survival and shoot growth when small explants were used; large explants gave only a slight increase in viability without shoot regrowth (Table 3). The survival of nondehydrated material was completely arrested at both temperatures with tips of both sizes. Dehydration at 26°–28°C favored tissue survival and resulted in significantly higher shoot growth from small explants.

Tissues with a relatively lower water content appear to have less surplus water for freezing into extracellular ice once freezing has begun and increased the concentration of solutes, with the result that the amount of water available for evaporation or sublimation is decreased (Ring and Danks 1994). We found that the temperature at which tissues were dehydrated before freezing affected viability and shoot formation after freezing. Results here confirm the shrinking effect of pre-treatment temperature on cryopreserved material, as shown by Finkle et al. (1985) in their work on cryoprotection.

Cassava genotypic response

The protocol followed here significantly influenced the tissue viability and shoot growth of the 15 cassava genotypes tested (Table 4). The highest responding genotypes in terms of viability also showed high shoot growth, except ‘MCol 1468’.‘MCol 22’ and ‘CM 922–2’, which are drought-tolerant varieties, and ‘MArg 2’, which is well-adapted to

subtropical conditions (M.A. El-Sharkawy personal communication) were the best responding genotypes. Varieties such as 'MDom 2', 'MVen 232', and 'MMex 71', with low tissue regrowth after freezing, also exhibited a gradual increase in bleaching that probably was caused by the toxicity of the cryoprotectants which, in turn, drastically reduced shoot growth (Table 4). Of the 15 cassava genotypes tested, 8 displayed an intermediate to high response in terms of shoot growth (i.e., 10–52%), while the shoot-tips of 3 varieties responded poorly and 4 did not form shoots at all.

All of the cassava varieties tested showed 100% tissue culture response, i.e., growth in 4E medium without preculture, cryoprotection and freezing (Table 4). Supplementary work carried out recently in our laboratory showed that the highly responding genotypes 'MCol 22' and 'CM 922–2' (Table 4) maintained a 100% response after preculture and cryoprotection but that freezing caused shoot regrowth to decrease to around 50%. Intermediate genotypes 'MEcu 48' and 'MPar 193' maintained 100% response after pre-culture, but cryoprotection caused shoot growth to decrease to 40%, and freezing to 15%. Shoot growth of poorly responding genotypes 'MVen 232' and 'MMex 71' decreased to 40% after pre-culture and to 3–5% after cryoprotection, leaving very little or no shoot-tip survival at all after freezing. Thus, while the freezing protocol *per se* can affect shoot regrowth, the more recalcitrant genotypes were affected mainly by the pre-culture and cryoprotection steps prior to freezing.

Recently we have evaluated the effect of modifying the preculture conditions by using lower sorbitol and DMSO concentrations (e.g. treatments 1.1 and 1.2; 4.1 and 4.2, Table 2) with some of the most recalcitrant varieties ('MVen 232' and 'MMex71'). Dramatic shoot regrowth improvements were obtained (18–20%), suggesting that the response of cassava varieties to cryopreservation can be improved. Results showed that pre-culture media containing high concentrations of osmotic agents for long periods reduce viability and shoot regrowth, even prior to freezing. Reducing the water content of small shoot tips resulted in higher viability and shoot regrowth after freezing when dehydration was carried out at 26°–28°C rather than at 0°C.

Using the methodology described here, we obtained repeatedly 50–70% of shoot growth and complete-plant formation with several cassava cultivars (Fig. 1A, B). Once shoot growth from shoot-tip explants occurred, rooting was readily achieved in 17N medium (Roca 1984). The varietal response of cassava to cryopreservation suggested that the edaphoclimatic adaptation of the cultivar may play a role in this response.

The observed differences in genotype response might be due to the steps before (pre-culture and cryoprotection) and/or after (re-culture) freezing. Once the various steps in the cryopreservation protocol have been standardized, efforts will be focused on the technical and logistical aspects of establishing an in vitro base gene bank in LN. Survival of cassava shoot tips after freeze-storage periods longer than 3 h will be tested. The genotypic stability of plants grown from cryopreserved shoot tips will be monitored, and the cost effectiveness of cryopreservation technology will be evaluated and compared with other technologies used at CIAT for the ex situ conservation of cassava genetic resources.

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