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CRYOPRESERVATION OF ZYGOTIC EMBRYOS OF *COFFEA* SPP.

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SUMMARY: Zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta at various maturity stages were successfully frozen in liquid nitrogen after partial dehydration in the air current of a laminar flow cabinet followed by rapid freezing. The optimal desiccation period was 0.5 hour. Survival was greater with embryos at an intermediate stage of maturity and ranged from 41.6% (28.9% water content) for *C. canephora* to 95.8% (15.8% water content) for *C. arabica*.

KEY WORDS: *Coffea*, zygotic embryos, cryopreservation, desiccation, germplasm storage, plant recovery.

INTRODUCTION

Seeds are generally used for germplasm storage. Seeds that resist high levels of desiccation and low temperatures without suffering damage (orthodox seeds) can be stored for long periods in seed genebanks with practical and economical results (1). However, there are seeds which cannot whithstand these storage conditions without rapidly loosing viability or being killed. These are the so called recalcitrant seeds (2, 3).

Coffee seeds have been considered recalcitrant based on their storage behaviour (4, 5, 6, 7). However, results from several studies showed that they did not behave as truly recalcitrant, since they resisted to relatively high levels of desiccation but viability loss increased with reduction in moisture level or temperature (8, 9, 10, 11, 12). Ellis and coworkers (8) suggested a new category of seed storage behaviour intermediate between orthodox and recalcitrant, which would include those seeds that as coffee can be dried but are injured by low temperature.

High germination rates could be maintained for 1 or even 2 years when coffee seeds were stored at about 10% moisture content and 15°C (8, 11), but no effective procedure has been described for long-term storage of these seeds.

Cryopreservation (liquid nitrogen, -196°C) has been shown as a feasible method for long-term storage of germplasm. Once the material is frozen all metabolic

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functions cease and no cell deterioration or mutation occurs, therefore genetic stabilitity of the stored material is maintained (13). This technique is specially useful for the long-term preservation of all those troublesome species which cannot be maintained under conventional storage methods.

For coffee germplasm preservation, cryopreservation can be useful as an alternative to field collections providing safe and economic long-term storage. Some preliminary attempts to cryopreserve coffee have been performed. Becwar and co-workers (7) tried to cryopreserve seeds of *C. arabica*. The results showed that even though seeds tolerated dehydration to as low as 8% moisture content, they did not withstand freezing in liquid nitrogen. In addition, successful cryopreservation of somatic embryos was reported for *C. arabica* (13) and *C. canephora* (14). The embryos were treated with high sucrose concentrations and slow freezing to -40°C prior to immersion in liquid nitrogen.

In the present study, the ability of zygotic embryos of coffee to withstand freezing in liquid nitrogen was examined. Embryos isolated from fruits at three different maturity stages were successfully cryopreserved following a dehydration pretreatment.

MATERIALS AND METHODS

Zygotic embryos of *C.arabica* cv Caturra, *C. canephora* and the hybrid arabusta (*C. arabica* x *C. robusta*) were used. They were extracted from fruits at three different maturity stages, based on skin coloration: green fruits, collected 2 months before harvest, yellow fruits, collected about 4 days before they turned red and red fruits ready to harvest. The fruits were disinfected by washing them with tap water and soap, 70% ethanol for 15 min and rinsing 3 times with sterile water. Embryos were isolated under aseptic conditions. They were cultivated on a medium containing the mineral solution and the vitamins of Murashige & Skoog (15), 30 $g.l^{-1}$ sucrose, 1 $mg.l^{-1}$ benzyladenine and 2 $g.l^{-1}$ gelrite. The pH was adjusted to 5.7 before autoclaving. Gibberellic acid (GA_3) at various concentrations was added in the recovery medium during some experiments.

For cryopreservation, the embryos were placed on small pieces of filter paper and dehydrated under the sterile air of a laminar flow cabinet for increasing lengths of time (0 to 2 hours). At the end of each dehydration period, samples were taken to determine the water content of the embryos. Freezing was carried out in 2 ml cryovials by direct immersion in liquid nitrogen. After a minimum of one hour storage at -196°C, the embryos were rewarmed by plunging the cryotubes for 60 to 90 s in a 40°C water-bath and then transferred onto the culture medium for recovery. The cultures were kept under low light (2 $\mu E.m^{-2}.s^{-1}$, 12h/24 photoperiod) for two days and then placed under normal light conditions (44 $\mu E.m^{-2}.s^{-1}$, same photoperiod) for germination and further development. The culture temperature was 27 ± 1°C.

Freezing results are the average of 3 experiments with 3 replicates of at least 12 embryos each. Survival was observed 4 weeks after freezing. At that time, living embryos had produced their first pair of leaves.

RESULTS

Untreated zygotic embryos extracted from yellow and green fruits showed 100% germination (Table 1 and 2). Water content varied with the maturity stage of the fruits from which the embryos were isolated (64% from green fruits, 42% from yellow fruits).

Embryos of coffee resisted freezing in liquid nitrogen only when dehydrated to water contents between 28.9% (Table 3) and 7.8% (Table 2). However, the rate of survival depended on the maturity stage of the fruits at the time embryos were isolated and on the water content of the embryos at the moment of freezing.

Embryos from immature (green) fruits of *C. arabica* were sensitive to air dehydration (Table 1), since after 30 min desiccation, survival decreased to 80% and fell to 52% after one hour desiccation. Embryos isolated from intermediate maturity (yellow) fruits of *C. arabica* showed 100% germination after 0 and 30 min desiccation (around 16% water content), but the germination rate decreased rapidly (to 55% and lower) when the dehydration period was increased (Table 2). Survival in liquid nitrogen was observed for desiccation durations ranging between 0.5 hour (16.4% water content) and 1.5 hours (7.8% water content). No survival was obtained for dehydration periods shorter than 0.5 hour (data not shown) and longer than 1.5 hours.

Even though embryos from mature (red) fruits of coffee dehydrated using the same process showed 100% survival after cryopreservation, contamination problems did not allow recovery of plantlets. At this maturity stage many fruits are burst or damaged by bacteria or by insects and even when using a stronger disinfection procedure, sanitary problems impeded the use of this material for conservation experiments.

In addition to *C. arabica*, freezing experiments were conducted with *C. canephora* and the hybrid arabusta. After 0.5 hour desiccation, embryos extracted from yellow fruits of *C. canephora* and arabusta had similar moisture contents around 28% (Table 3), whereas that of *C. arabica* was lower. Survival of desiccated control embryos was 100% with the three species. However, after cryopreservation, high survival rates (83 to 95%) were obtained with embryos of *C. arabica* and arabusta, whereas survival of *C. canephora* embryos was less than half of that of the two other species.

The relatively low survival rate of immature embryos of *C. arabica* (extracted from green fruits), desiccated for the optimal duration and frozen in liquid nitrogen, could be increased to levels comparable to that observed with more mature embryos (yellow fruits) by adding 10 to 100 $mg.l^{-1}$ GA_3 to the recovery medium (Table 4). However, their further development into whole plantlets was the most satisfactory after a treatment with 10 $mg.l^{-1}$ GA_3.

In all experiments, the further development of cryopreserved embryos was only slightly delayed, compared to that of unfrozen controls. In the case of embryos of *C. arabica* extracted from green fruits, this delay was reduced by the addition of 10 $mg.l^{-1}$ GA_3. Fully developed plantlets were obtained with all species.

DISCUSSION/CONCLUSION

During this study, an efficient cryopreservation method was set up for zygotic embryos of *Coffea*, since 41 to 96% survival of frozen embryos could be obtained, depending on the species.

This process is characterized by its simplicity, since the embryos were only submitted to a partial desiccation before their direct immersion in liquid nitrogen. It is similar to that used with zygotic embryos of several species (17). Indeed, zygotic embryos are usually desiccated in the air current of a laminar flow cabinet, down to optimal water contents of 10 to 16%, depending on the species. A comparable value was observed with embryos of *C. arabica* only. The embryos of *C. canephora* and arabusta are larger and may thus dehydrate more slowly. Therefore, their optimal dehydration period may be longer than that of *C. arabica* (0.5 hour).

Higher survival rates were obtained with more mature embryos, i.e. extracted from yellow fruits, compared with that extracted from green fruits. This may be due to the fact that immature embryos generally need to be grown on more sophisticated culture media (18) and not to a greater extent of damages caused to immature embryos by freezing in liquid nitrogen, in comparison to that observed with more mature ones. Indeed, the addition of GA_3, which is known to promote the growth of immature embryos (19) in the recovery medium allowed to increase the survival rate of immature embryos of *C. arabica* to a level comparable to that of more mature ones.

The extension of the dehydration period from 1.5 to 2 hours did not result in a further decrease in the water content of the embryos of *C. arabica* but induced a total loss of survival in cryopreserved embryos. This could be due to an increase in the damages caused to the tissues by this prolonged dehydration, as shown by the reduction in the survival rate observed with control embryos.

In conclusion, this preliminary study showed that it was possible to successfully cryopreserve zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta and to obtain the development of plantlets from frozen embryos. However, additional work is needed to improve these results: longer desiccation periods should be experimented with embryos of *C. canephora*. The further development *in vivo* of the plantlets should be monitored and the present technique refined and experimented with other *Coffea* species. The use of cryopreservation for the long-term preservation of coffee genetic resources may thus be foreseable in the near future.

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Table 1: Moisture content and survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica* isolated from green fruits.

Dehydration (hours)	Moisture content (%)	Survival (%)	
		-LN	+LN
0.0	64.2±1.2	100.0±0.0	0.0±0.0
0.5	21.2±1.9	80.0±0.0	50.0±4.0
1.0	10.1±0.9	52.6±7.4	34.2±5.4
1.5	7.8±1.3	25.0±5.3	13.9±2.7
2.0	5.0±2.1	5.5±2.7	0.0±0.0

Table 2: Moisture content and survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica* isolated from yellow fruits.

Dehydration (hours)	Moisture content (%)	Survival (%)	
		-LN	+LN
0.0	41.9±0.5	100.0±0.0	0.0±0.0
0.5	16.4±0.4	100.0±0.0	96.0±4.0
1.0	8.4±0.3	55.5±2.8	41.6±6.2
1.5	7.8±1.1	27.7±2.7	19.4±2.8
2.0	7.7±1.5	8.3±0.0	0.0±0.0

Table 3: Survival after dehydration (-LN) or dehydration and freezing in liquid nitrogen (+LN) of zygotic embryos of *C. arabica*, *C. canephora* and the hybrid arabusta isolated from yellow fruits and dehydrated for 0.5 hour.

	Moisture content (%)	Survival (%)	
		-LN	+LN
C. arabica	15.8±0.26	100.0±0.0	95.8±4.1
C. canephora	28.9±2.9	100.0±0.0	41.6±2.2
arabusta	27.3±3.3	100.0±0.0	83.6±3.6

Table 4: Effect of GA_3 concentration in the recovery medium on the survival rate of embryos of *C. arabica* extracted from green fruits, dehydrated for 0.5 hour and frozen in liquid nitrogen.

GA_3 ($mg.l^{-1}$)	Survival (%)
0.0	52.1±4.2
10.0	75.0±0.0
33.3	75.0±8.3
100.0	83.3±8.3