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# CRYOPRESERVATION OF *Citrus aurantifolia* SEEDS AND EMBRYONIC AXES USING A DESICCATION PROTOCOL

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**Abstract**

The desiccation and freezing tolerance of seeds, with and without testas, and embryonic axes of *Citrus aurantifolia* were investigated. Seeds were desiccated with silica gel, under the laminar air flow cabinet or by placing them on a laboratory bench. Whatever the desiccation method employed, survival before and after cryopreservation was higher for seeds without testas. When freezing intact seeds, the highest survival percentage (41.3 %) was achieved after desiccation to 7.3 % moisture content (fresh weight basis) on the laboratory bench. Survival of seeds cryopreserved without testas could reach up to 85 % after desiccation under the laminar air flow cabinet or on the laboratory bench, corresponding to moisture contents of 7.1 and 4.5 %, respectively. After desiccation with silica gel, survival reached a maximum of 60.0 %, for a seed moisture content of 3.3 %. Survival of control embryonic axes was high (80-100 %) whatever the sucrose concentration in the preculture medium and the duration of the desiccation period. After cryopreservation, no survival was noted with embryonic axes, which had not been precultured nor desiccated. Survival of non-desiccated embryonic axes after cryopreservation increased progressively in line with increasing sucrose concentrations in the preculture medium, from 7.5 % with 0.1 M sucrose to 77.5 % with 0.7 M sucrose. Survival of desiccated and cryopreserved embryos was always high, whatever the preculture treatment and desiccation period, ranging from 55.8 % to 92.5 %.
**Keywords:** *Citrus aurantifolia*; seed; embryonic axis; desiccation; cryopreservation.

## INTRODUCTION

Citrus seeds have been considered recalcitrant for many years (9). However, following an initial report demonstrating that *Citrus limon* seeds were tolerant to desiccation (13),

additional investigations were performed on the response of seeds of other citrus species and related genera to desiccation and cryopreservation. These studies revealed different responses between species, ranging from high desiccation tolerance and survival after freezing, as in the case of *C. limon* to high desiccation sensitivity and absence of survival after freezing, as in the case of *Poncirus trifoliata* (5, 6, 7). Citrus seeds are generally cryopreserved by direct immersion in liquid nitrogen (LN) after partial desiccation using air- or silica gel-drying. Seeds of species which are highly tolerant to desiccation, such as *C. limon, C. aurantium* and *C. aurantifolia*, produce high survival percentages (11, 12, 13, 16, 19). By contrast, seeds which are less tolerant to desiccation, such as those of *C. sinensis, C. deliciosa, C. sinensis x P. trifoliata* and *C. halimii*, require careful monitoring of drying to avoid desiccation beyond a critical value, which varies among species, in order to achieve intermediate survival percentages after desiccation and freezing (14, 17). In the case of species whose seeds are highly sensitive to desiccation, such as *P. trifoliata* and *C. hystrix*, no survival after cryopreservation has yet been achieved (16, 19, 20). It has also been shown recently that seeds of *C. madurensis*, which display intermediate desiccation tolerance, could withstand cryopreservation after pregrowth on medium with high sucrose concentration followed by partial desiccation and rapid freezing (10).

Attempts have also been made to investigate the tolerance to desiccation and suitability for cryopreservation of embryonic axes excised from citrus seeds. The limited information published indicates that, as observed with most recalcitrant and intermediate species, citrus embryonic axes are generally more tolerant to desiccation than whole seeds and that they can also be cryopreserved by direct immersion in LN after partial drying (4, 8). Survival of embryonic axes after cryopreservation was high with *P. trifoliata, C. aurantifolia* and *C. medica* (16, 19) and intermediate with *C. halimii* and *C. hystrix* (14). However, no survival was achieved with *C. mitis* embryos (15). Embryonic axes of *C. madurensis* could be successfully cryopreserved using both a vitrification (1, 2) and an encapsulation-dehydration protocol (3).

Numerous factors are involved in the tolerance of non-orthodox seeds to desiccation (17). Among these factors, the drying rate seems to play a critical role in the retention of viability of seeds or excised axes. The more rapidly dehydration can be achieved, the lower is the water content to which the seeds or axes can be dried before viability is lost.

South East Asia, including Malaysia, is known to be the centre of diversity and distribution of citrus, especially members of the *Aurantioideae* subfamily, which includes *C. aurantifolia*, commonly known as lime, sour lime or common lime (7, 16). The fruit is used mainly to flavor food in nearly every home in South East Asia, but also to prepare drinks and it has a variety of medicinal applications.

In the present study, we compared the sensitivity of seeds of *C. aurantifolia*, with and without testa, to desiccation using different methods and to cryopreservation. We also observed the effect of sucrose preculture and desiccation on the survival of excised embryonic axes after cryopreservation.

## MATERIALS AND METHODS

*Plant material*

Fruits of *C. aurantifolia* were collected from the field collections of UKM (Universiti Kebangsaan Malaysia) and MARDI (Malaysian Agricultural Research and Development Institute). Experiments were performed in the laboratories of the School of Bioscience and Biotechnology in UKM, where the average relative humidity was around 50 %. Seeds (15-25 seeds per fruit) were extracted and seed moisture content (MC, fresh weight basis) was determined with 20 individual seeds using the oven-drying method (103 °C for 24 h). For MC

determination, each seed (length 8.5 ± 0.7 mm, width 5.0 ± 0.5 mm, thickness 3.5 ± 0.3 mm) was cut into 3-5 pieces before drying.

*Desiccation and cryopreservation of whole seeds*

Seeds were surface-sterilized with absolute ethanol for 2 min, then placed for 20 min in 20 % commercial bleach (0.54 % active chlorine) with a few drops of Tween 20 on a rotary shaker. They were then rinsed 3-5 times with sterile distilled water. The testa of seeds was then removed, or intact seeds were used for further experiments.

Seeds were desiccated using three methods: they were placed i) in an air-tight container (diameter 12 cm, height 9 cm), with 70 g silica gel for 200 seeds; ii) in the laminar air flow cabinet; or iii) on a laboratory bench at room temperature. Desiccation periods ranged between 0 and 8 days. Seed MC during desiccation was measured gravimetrically (by placing the seeds in an oven at 103°C for 24 h) at the end of each desiccation period using 20 intact seeds or 10 seeds without testas. Seed MC was expressed on a fresh weight basis.

For cryopreservation, seeds were wrapped in aluminum foil envelopes, immersed directly in LN and stored at –196°C for 24 h. They were then rewarmed for 5 min in a water-bath at 40°C, plated on MS medium with 0.3 M sucrose for 1 day to reduce the osmotic shock which may occur during rehydration, sown in moist sand in plastic pots in the greenhouse and cultured under natural temperature and light conditions. Observations were made twice a week during the first four weeks, then once a week up to the eighth week. Seeds which produced a morphologically normal seedlings were considered viable.

Experiments were performed with 20 intact seeds and 10 seeds without testas per experimental condition and replicated four times. Data were arcsin transformed and analyzed by ANOVA using Duncan's multiple range test for data comparison. In the Tables, mean survival percentages are presented with their standard deviation.

*Desiccation and cryopreservation of excised embryonic axes*

Seeds were sterilized as described previously. Embryos (1-2 mm long) were excised aseptically from the seeds by removing the testas; axes were then separated from the cotyledons with a scalpel blade. Axes were then precultured for 24 h on basal MS medium (14) supplemented with various sucrose concentrations (0.1 to 0.7 M), 7g $l^{-1}$ Difco agar and 0.3mg $l^{-1}$ benzylaminopurine (BAP). They were then desiccated for 1 to 3 h in the laminar air flow cabinet, wrapped in aluminium foil envelopes and frozen rapidly by direct immersion in LN, where they were kept for 24 h. The MC of embryonic axes was determined gravimetrically, as described previously, at the end of each desiccation period using 10 embryos. They were rewarmed for 5 min in a water-bath at 40°C, placed on MS medium with 0.3 M sucrose for 1 day, then transferred on to standard MS medium with 0.1 M sucrose and 0.1 mg $l^{-1}$ BAP at 25 ± 1°C under a 16 h light/8 h dark photoperiod with a light intensity of 25 µmol $m^{-2}$ $s^{-1}$. Embryonic axes were recorded as surviving when they developed into whole seedlings.

Experiments were performed with 10 embryonic axes per experimental condition and replicated four times. Data were arcsin transformed and analyzed by ANOVA using Duncan's multiple range test for data comparison. In the Tables, mean survival percentages are presented with their standard deviation.

## RESULTS

Tables 1-3 present the moisture contents and germination percentages of *C. aurantifolia* seeds with and without testa following desiccation with the various methods tested and cryopreservation.

In all cases, the initial MC was higher for intact seeds than for seeds without testas (Tables 1-3). When desiccation was performed with silica gel (Table 1), desiccation was faster for seeds without testa but seeds with and without testas reached similar MCs from 4 days of desiccation onwards. Survival of control intact seeds decreased from 82.5 % without desiccation to 21.5 % after 8 days of desiccation. After cryopreservation, only limited survival was achieved after 2 (16.5 %) to 8 days (15.0 %) of desiccation, with an optimum of 24.2 % after 3 days, corresponding to a seed MC of 6.0 %. In the case of seeds without testas, survival of desiccation controls decreased from 92.5 % without desiccation to 57.5 % after 8 days of desiccation. Survival of cryopreserved seeds was achieved after 1 to 8 days of desiccation. Survival was highest after 2 to 4 days of desiccation, for MCs ranging between 7.7 and 3.6 %, and varied from 55.3 to 60.0 %.

Table 1. Effect of desiccation period with silica gel on the moisture content (%, fresh weight basis) and on the survival (%, ±SD) of control (-LN) and cryopreserved (+LN) *Citrus aurantifolia* seeds, with or without testas. Values followed by the same letter within treatment (-LN, or +LN) are not significantly different at the 0.05 probability level based on Duncan's test.

| Desiccation period (days) | With testas | | | Without testas | | |
|---|---|---|---|---|---|---|
| | Seed moisture content (%) | Survival (%) | | Seed moisture content (%) | Survival (%) | |
| | | -LN | +LN | | -LN | +LN |
| 0 | 53.1±3.6 | 82.5±2.9$^{a}$ | 0 | 43.4±5.7 | 92.5±5.0$^{a}$ | 0 |
| 1 | 29.1±2.6 | 90.0±4.1$^{a}$ | 0 | 21.7±5.0 | 87.5±9.6$^{a}$ | 32.5±9.6$^{c}$ |
| 2 | 15.5±2.4 | 65.0±4.1$^{b}$ | 16.5±8.5$^{a}$ | 7.7±1.8 | 85.0±5.8$^{a}$ | 57.5±9.6$^{a}$ |
| 3 | 6.0±0.8 | 40.3±5.0$^{c}$ | 24.2±10.0$^{a}$ | 3.3±0.3 | 72.5±5.0$^{b}$ | 60.0±8.2$^{a}$ |
| 4 | 3.9±0.4 | 31.5±8.5$^{d}$ | 18.7±9.5$^{a}$ | 3.6±0.5 | 100$^{a}$ | 55.3±5.0$^{a}$ |
| 8 | 3.6±0.9 | 21.5±2.5$^{d}$ | 15.0±14.1$^{a}$ | 2.4±0.3 | 57.5±5.0$^{c}$ | 45.0±5.8$^{b}$ |

During desiccation in the laminar air flow cabinet, the decrease in seed MC was faster for seeds without testas (Table 2). Survival of intact control seeds decreased from 88.8 % without desiccation to 6.1 % after 8 days of desiccation. After cryopreservation, survival was noted only after 2 and 3 days of desiccation, and the survival percentages achieved were intermediate, ranging between 32.8 and 36.3 %, corresponding to seed MCs of 15.8 and 7.9 %, respectively. Survival of control seeds without testas varied between 92.5 % without desiccation and 62.2 % after 8 days of desiccation. After cryopreservation, survival of seeds without testas was achieved for desiccation periods between 1 and 8 days. The highest survival percentages were obtained after 2 (85.0 %) and 3 (82.5 %) days of desiccation, corresponding to seed MCs of 7.1 and 6.5 %, respectively.

When desiccation was performed by placing the seeds on a laboratory bench, dehydration of seeds without testas was faster than that of intact seeds during the first 4 days (Table 3). Survival of control intact seeds varied from 91.3 % without desiccation to 25.0 % after 8 days of desiccation. After cryopreservation, survival was achieved for desiccation periods ranging between 2 and 8 days, with percentages between 20 % (2 days) and 41.3 % (6 days). In the case of seeds without testas, survival of controls remained very high (82.5-97.5 %) whatever the desiccation period and no significant differences between desiccation durations were noted. After cryopreservation, very high survival percentages, ranging between 72.5 and 85.0 %, were achieved for desiccation periods between 2 and 8 days, with seed MCs between 7.2 % (2 days) and 4.5 % (8 days).

Table 2. Effect of desiccation period in the laminar air flow cabinet on the moisture content (%, fresh weight basis) and on the survival (%, ± SD) of control (-LN) and cryopreserved (+LN) *Citrus aurantifolia* seeds, with or without testas. Values followed by the same letter within treatment (-LN, or +LN) are not significantly different at the 0.05 probability level based on Duncan's test.

| Desiccation period (days) | With testas | | | Without testas | | |
|---|---|---|---|---|---|---|
| | | Survival (%) | | | Survival (%) | |
| | Seed moisture content (%) | -LN | +LN | Seed moisture content (%) | -LN | +LN |
| 0 | 53.7±7.1 | 88.8±7.5$^{a}$ | 0 | 41.5±3.5 | 92.5±9.6$^{a}$ | 0 |
| 1 | 28.7±2.8 | 87.5±2.9$^{a}$ | 0 | 15.5±3.1 | 90.0±8.2$^{a}$ | 32.5±5.0$^{c}$ |
| 2 | 15.8±2.8 | 73.8±8.5$^{b}$ | 36.3±6.3$^{a}$ | 7.1±1.0 | 92.5±5.0$^{a}$ | 85.0±5.8$^{a}$ |
| 3 | 7.9±0.4 | 45.0±4.1$^{c}$ | 32.8±9.6$^{a}$ | 6.5±1.3 | 87.5±5.0$^{a}$ | 82.5±5.0$^{a}$ |
| 8 | 6.6±1.4 | 6.1±5.0$^{e}$ | 0 | 3.0±0.5 | 62.2±5.0$^{b}$ | 59.7±10$^{b}$ |

Table 3. Effect of desiccation period on a laboratory bench on the moisture content (%, fresh weight basis) and survival (%, ±SD) of control (-LN) and cryopreserved (+LN) *Citrus aurantifolia* seeds, with or without testas. Values followed by the same letter within treatment (-LN, or +LN) are not significantly different at the 0.05 probability level based on Duncan's test.

| Desiccation period (days) | With testas | | | Without testas | | |
|---|---|---|---|---|---|---|
| | | Survival (%) | | | Survival (%) | |
| | Seed moisture content (%) | -LN | +LN | Seed moisture content (%) | -LN | +LN |
| 0 | 53.9±5.5 | 91.3±2.5$^{a}$ | 0 | 41.1±3.7 | 97.5±5.0$^{a}$ | 0 |
| 1 | 35.1±4.2 | 86.4±5.0$^{a}$ | 0 | 17.2±1.6 | 88.9±5.0$^{a}$ | 25.0±5.0$^{b}$ |
| 2 | 16.7±3.8 | 81.3±15.5$^{a}$ | 20.0±14.7$^{b}$ | 7.2±0.7 | 85.0±5.8$^{a}$ | 77.5±5.0$^{a}$ |
| 4 | 8.3±0.4 | 60.0±7.1$^{b}$ | 40.0±11.1$^{a}$ | 5.3±1.1 | 82.5±5.0$^{a}$ | 72.5±9.6$^{a}$ |
| 6 | 7.3±1.0 | 53.8±16.0$^{b}$ | 41.3±11.8$^{a}$ | 5.0±1.3 | 87.5±5.0$^{a}$ | 80.0±8.2$^{a}$ |
| 8 | 6.1±1.1 | 25.0±9.1$^{c}$ | 22.8±10.4$^{b}$ | 4.5±1.1 | 82.5±5.0$^{a}$ | 85.0±5.8$^{a}$ |

When comparing the results obtained for seeds with and without testas after desiccation using the three methods employed, it appeared that, in all cases, higher survival percentages were consistently achieved with seeds without testas, both before and after cryopreservation (Tables 1-3). In the case of seeds without testas, the optimal survival percentages were higher after desiccation in the laminar air flow cabinet and on the laboratory bench than with silica gel. Even though the optimal survival percentages after cryopreservation were similar (85 %, Tables 2 and 3) with the former two techniques, desiccation on the laboratory bench ensured comparable survival percentages for a broader range of desiccation durations, from 2 to 8 days, compared with 2 and 3 days of dehydration under laminar flow conditions.

Table 4. Effect of sucrose concentration in the preculture medium and of the duration of the desiccation period in the laminar air flow cabinet on the survival (%, ±SD) of control (-LN) and cryopreserved (+LN) *Citrus aurantifolia* embryonic axes. Values followed by the same letter within treatment (-LN, or +LN) are not significantly different at the 0.05 probability level based on Duncan's test.

| Sucrose concentration (M) in preculture medium | Desiccation period (h) | Moisture Content (%) | Survival (%) | |
|---|---|---|---|---|
| | | | -LN | +LN |
| 0 | 0 | 59.3±5.8 | 100[a] | 0[f] |
| | 1 | 11.4±2.9 | 92.6±5.0[a] | 90.0±5.0[a] |
| | 2 | 7.6±2.1 | 100[a] | 55.8±15.0[b] |
| | 3 | 7.1±1.8 | 100[a] | 66.7±10.0[b] |
| 0.1 | 0 | 33.0±4.4 | 97.5±5.0[a] | 7.5±9.6[e] |
| | 1 | 14.4±2.6 | 90.0±0.0[a] | 90.0±8.2[a] |
| | 2 | 7.3±1.2 | 92.5±5.0[a] | 92.5±5.0[a] |
| | 3 | 5.3±2.6 | 87.5±9.6[a] | 82.5±9.6[a] |
| 0.3 | 0 | 35.0±4.1 | 97.5±5.0[a] | 25.0±17.3[d] |
| | 1 | 14.5±2.1 | 90.0±8.2[a] | 85.0±5.8[a] |
| | 2 | 8.2±1.4 | 85.0±5.8[a] | 80.0±0.0[a] |
| | 3 | 6.9±1.5 | 85.0±5.8[a] | 82.5±5.0[a] |
| 0.5 | 0 | 34.4±6.0 | 97.5±5.0[a] | 35.0±23.8[c] |
| | 1 | 17.2±1.9 | 90.0±8.2[a] | 87.5±5.0[a] |
| | 2 | 7.2±0.8 | 82.5±5.0[a] | 80.0±0.0[a] |
| | 3 | 5.6±1.1 | 80.0±8.2[a] | 75.0±5.8[a] |
| 0.7 | 0 | 27.7±4.8 | 95.0±10.0[a] | 77.5±5.0[a] |
| | 1 | 13.4±2.1 | 90.0±8.2[a] | 82.5±15.0[a] |
| | 2 | 4.8±2.0 | 92.5±9.6[a] | 82.5±5.0[a] |
| | 3 | 3.9±1.4 | 90.0±8.2[a] | 77.5±5.0[b] |

Table 4 shows the MC and germination percentage of control and cryopreserved *C. aurantifolia* embryonic axes after preculture on media with different sucrose concentrations followed by desiccation. Desiccation of embryonic axes was rapid during the first hour, reaching MCs between 11.4 % (no preculture) and 17.2 % (preculture with 0.5 M sucrose), then slower during the last 2 hours, with the lowest MCs achieved between 3.9 % (preculture with 0.7 M sucrose) and 7.1 % (no preculture). Survival of embryonic axes after desiccation was high (80-100%) whatever the sucrose concentration in the preculture medium and the duration of the desiccation period. After cryopreservation, no survival was noted with embryonic axes that had beenneither precultured nor desiccated. Survival of non-desiccated embryonic axes increased progressively in line with increasing sucrose concentrations in the preculture medium, from 7.5 % with 0.1 M sucrose to 77.5 % with 0.7 M sucrose. Survival of desiccated and cryopreserved axes was always high whatever the preculture treatment and desiccation period, ranging from 55.8 % (no preculture, 2 h of desiccation) to 92.5 % (preculture with 0.1 M sucrose, 2 h of desiccation).

## DISCUSSION

In this paper, we showed that *C. aurantifolia* seeds without testas displayed higher survival before and after freezing than intact seeds, whatever the desiccation method employed (silica gel, laminar air flow cabinet or laboratory bench). Consideration of the results for intact seeds versus those with testas removed showed that, irrespective of the mode of drying employed, water loss over the first two days was more rapid for the latter. During this period, seeds would have been passing through moisture content ranges commensurate with metabolism-linked damage (17). It can be presumed from the present results for *C. aurantifolia* that the more rapidly this passage was achieved, the less the degree of such damage, as has previously been shown notably for seeds of *Ekebergia capensis* (18) and *Camellia sinensis* (21), both of which are desiccation-sensitive.

Survival after cryopreservation of seeds without testas was also achieved for a broader range of MCs, ranging from 21.7 % to 2.4 %. These results confirm the previous observations of King *et al.* (12) and Normah and Serimala (16) on the high tolerance to desiccation of *C. aurantifolia* seeds. In our study, the optimal survival percentage after cryopreservation was higher than that observed by Normah and Serimala (16), possibly because we employed seeds without testas, thus ensuring a higher freezing rate, thereby limiting intracellular damages. Higher survival was consistently achieved after desiccation in the laminar air flow cabinet and on the laboratory bench than with silica gel, even though the desiccation rates measured with the three methods employed were similar. In future experiments, it might be worth testing desiccation with saturated salt solutions, which should ensure very precise and reproducible desiccation conditions, provided that it is always performed at the same temperature.

In the case of excised embryonic axes, survival of non-frozen controls was high whatever the sucrose preculture treatment and the duration of the desiccation period. After freezing, survival of embryonic axes that had not been desiccated increased in line with the sucrose concentration of the preculture medium and was achieved for high embryonic axis MCs, ranging between 27.7 and 34.4 %. Sucrose has two modes of action: it partially dehydrates samples through osmotic effects; it is also absorbed by samples, thereby increasing the concentration of intracellular solutes (4). As regards embryonic axes which had been precultured with sucrose and desiccated, there was no clear effect of the preculture treatment and of the duration of the desiccation period. Survival was high provided that the MC of embryonic axes was lower than 27.7 %, and results were comparable to those obtained by Normah and Serimala (16) on the same material.

In conclusion, in view of the high tolerance to desiccation and cryopreservation of seeds and excised embryonic axes of *C. aurantifolia*, cryopreservation can be readily employed for the safe and cost-effective long-term conservation of genetic resources of this species.

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