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Research article

Ultrastructural studies in Litchi (*Litchi chinensis* Sonn.) after desiccation and freezing

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Abstract: Recalcitrant seeds are remarkably short-lived and therefore are difficult to be successfully stored with respect to the ex situ conservation. Litchi seed embryonic axes were desiccated before cryopreserved in liquid nitrogen (direct immersion). Microscopic investigations were undertaken on Litchi embryonic axes in response to the desiccation and freezing sensitivity. Evidences shows that cryopreservation has a disruptive effect at the cellular level in Litchi.
Keywords: Cryopreservation - Desiccation - Embryonic axes - Litchi - Recalcitrant.

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INTRODUCTION

Litchi (*Litchi chinensis* Sonn.) belongs to the family Sapindaceae and subfamily Nepheleae which has about 125 genera and more than 1,000 species (Chapman 1984). Being a originated in the area of southern China, northern Viet Nam and Myanmar, lychee has long history of domestication. It is now cultivated in most of the tropical and sub-tropical regions of the world. The genus Litchi has two species, *Litchi philippinensis* Radlk. and *Litchi chinensis* Sonn. (Syn. *Nephelium Litchi*). The former is a wild plant grown largely in the Philippines and not of commercial importance, except when used as rootstock (Menzel 1983). Litchi fruits consist of aril as a juicy edible part, seed and peel. Most of the litchi produced is consumed as fresh fruits, and also used as frozen, canned or dried fruits. Litchi seeds are composed of polysaccharides (40.7%), proteins (4.93%), crude fibers (24.5%), oil (3.2%) and some minerals (Koul & Singh 2017) and most recently Punia & Kumar (2021) have been published comprehensive compilation of literature on Litchi.

On the account of conservation of plant genetic resources, the seed is the most widely used form. Many tree species produce seeds which do not dry as they mature and usually shed with relatively high moisture content. Being a recalcitrant type of seed, Litchi seeds are of short viability and begin losing viability within a day of removal from the fruit (Ray & Sharma 1987). Attempts made to store litchi seeds at low temperature (5°C) showed that about 36% viability could be maintained up to 100 days. Seeds are highly desiccation-sensitive and lose viability at about 27% moisture content. In the present paper, the authors discuss the ultra-structural studies on changes that occurred during desiccating and freezing of embryonic axes of the Litchi seed.

MATERIALS AND METHODS

Two varieties namely Calcuttia and Rose-scented were selected for studies on desiccation and freezing (cryopreservation). Suitable trees of these varieties were tagged from the day of anthesis. Fruits at different stages of maturity (as measured by days after anthesis) were harvested during the month of May and June. The fruits were brought to the laboratory within 2 days of harvest. The seeds were extracted after removing the aril on the day of experimentation and used up the same day.

Desiccation and freezing (cryopreservation)

Axes were excised aseptically under the laminar flow. For these studies, 80 DAA seeds were chosen. Fresh

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embryonic axes were spread over sterile filter paper in batches of 50 and gradually desiccated for 60 min and 80 min under the laminar flow. The desiccated embryonic axes were sealed in polypropylene cryovials of 1–2 ml capacity and frozen using rapid freezing, *i.e.* direct plunging in liquid nitrogen (-196°C). After 24 hrs to 48 hrs of storage, the samples were rapidly thawed in a water bath at 37°C and subsequently used within 15 min for regeneration and other analysis.

Ultra-structural study

Transmission electron microscopic studies were undertaken with fresh, desiccated and cryopreserved embryonic axes at two maturity stages. For this, the 1–2 mm terminal radical portions of 5–6 axes from each sample were cut and fixed in Karnowsky fluid overnight at 40°C, followed by 2 hrs at room temperature. Samples were thoroughly washed in 0.1 M phosphate buffer (pH 7.2) and osmiccated in 0.5% osmium tertaoxide for 5 hrs. Dehydration was done in graded series of ethanol. Propylene oxide was used at the last stage of dehydration and as an intermediate solvent which facilitated better infiltration. Vacuum infiltration was routinely carried out at each stage before embedding in epon-araldite mixture. Ultra-thin sections were mounted on grids and stained with lead citrate and uranyl acetate. In all samples, perenchymatous and meristematic cells were examined ulrastrcturally using Philips Transmission Electron Microscope Model CM-10 and CM-300.

RESULTS AND DISCUSSION

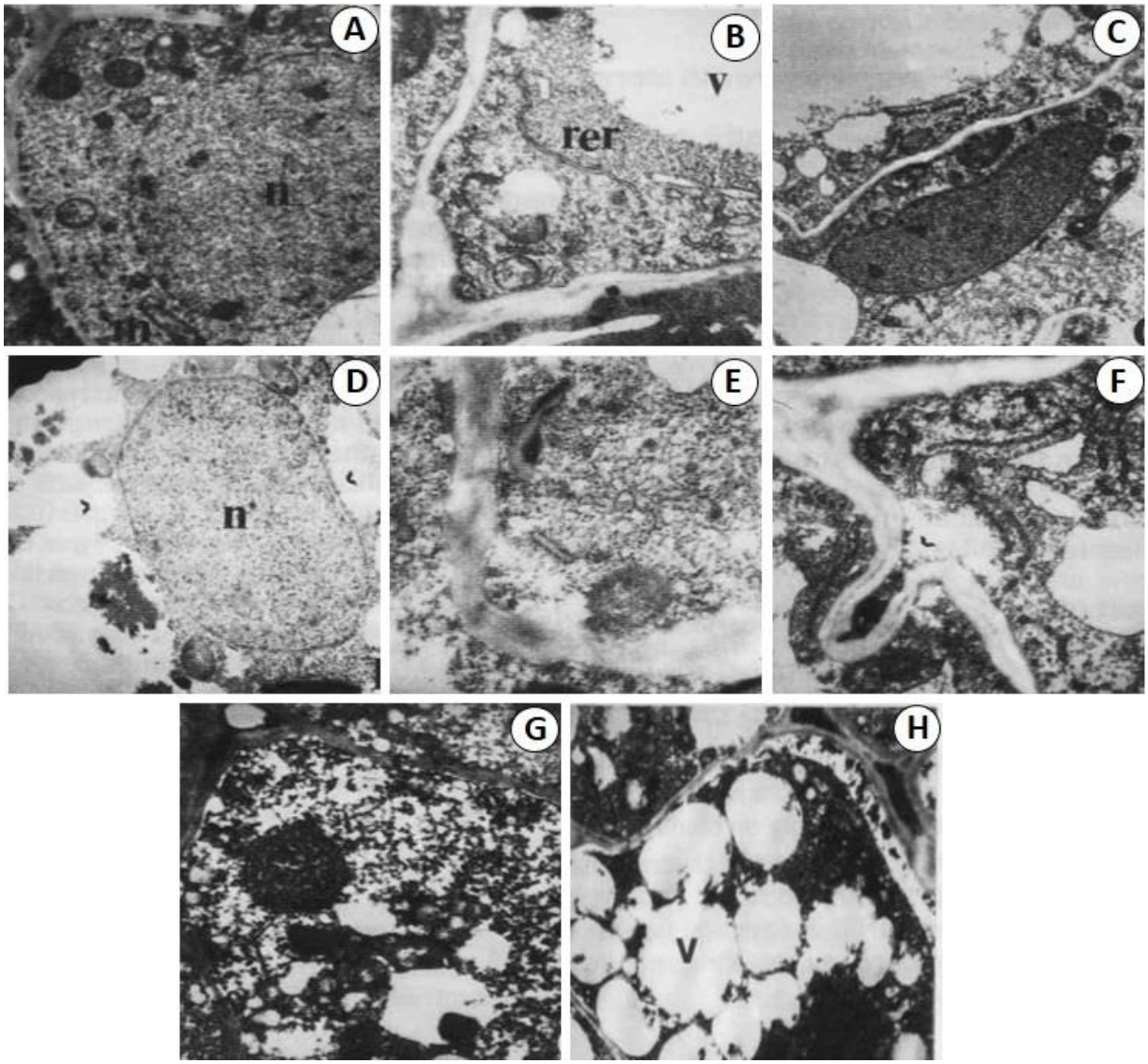

Figure 1. Transmission Electron Microscope studies in embryonic axes of Litchi var. rose scented. **A,** Parenchymatous cell of fresh axes with round nucleus; **B,** Cell with long profiles of rER of fresh axes; **C,** Cell structure of desiccated axes with elongated nucleus and other cell inclusions; **D,** Desiccated cell showing scattered cytoplasm with degenerating cell organelles; **G,** Completely collapsed cell at 14% moisture, only a mass of cytoplasm; **H,** Cell after desiccation and cryopreservation, complete collapse of cell inclusions and high degree of vacuolation near the cell wall. [n = Nucleus, rer = Rough Endoplasmic Reticulum, v = Vacuole; Scale: A- X8400; B, C- X10, 800; D, E- X16, 400; F, G, H- X5800]

Theoretically, prolonging the shelf life of the fruit is hampered by membrane integrity. Due to the low storage cost and ease of seed handling makes seed the most preferred part for ex situ germplasm conservation (Berjak *et al*. 2000). Orthodox seeds are acquiescent to conventional gene bank storage practices. However, recalcitrant seeds are constrained by desiccation and freezing sensitivity, therefore embryo and embryonic axes are an alternative option to cryopreserve the recalcitrant species (Bajaj 1985).

We attempted cryopreservation of embryonic axes from recalcitrant seeds of Litchi varieties. The aim was to study the possible damages to the isolated embryonic axes and subsequent viability after cryo exposure. In fresh embryonic axes, radical cells showed round nucleus with nucleolus (Fig. 1A). Long profiles of endoplasmic reticulum with ribosomes and spherical and elongated to elongated mitochondria of different stages were present (Fig. 1B). Plastids were apparent, occasionally full of starch granules. Lipid globules were scattered near the cell walls and large protein bodies were visible with dense to shallow matrix. Chandel *et al*. (1995) has also been reported similar biochemical evidences in Tea, Cocoa and Jackfruit while exposing to desiccation and cryopreservation. Dictyosomes were occasionally observed. Parenchymatous cells of radical of axes desiccated to 18% moisture content showed nucleus with intact nuclear envelope but the shape of nucleus became elongated to irregular (Fig. 1C). Cytoplasm became scattered due to formation of large numbers of vacuoles and degeneration of cell contents. Generally, embryonic axes behave as a seed in conservation practices. This is attributed to their more tolerance potential than seed. Our study further supports Pukacka & Ratajczak (2007).

In cryopreserved axes desiccated to 14% moisture content, cell walls were found folded and also disrupted at many places, which led to changes in the shape of cells (Fig. 1H). Steponkus (1984) has also been reported the similar types of symptoms of freezing damage in cytoplasmic membranes of plant tissues. Therefore, membrane permeability can be useful to measure the desiccation tolerance of embryonic axes. Cytoplasm became fragmented and scattered near the cell wall. Cytoplasm and cell inclusions became highly electron dense (Fig. 1H). Nucleus was observed only in form of dense and irregular nuclear material. Mitochondria, rER, plastids and other membrane bound cell organelles were completely collapsed and were seen scattered in cells. Other storage materials could not be identified in the collapsed cells. The apparent absence of ice crystal from the interior of ER and other organelles could be an important factor rate of success in cryo- survival of Litchi embryonic axes. Similar observations has been made by Normah & Makeen (2008) which determined that excised embryo and embryonic axes are more tolerant to desiccation and subsequent exposure to cryopreservation than whole seeds. Further, it is needed to experiment on somatic embryogenesis for mass production and genetic improvement of Litchi.

CONCLUSION

In conclusion, the present study highlights the possible effects of desiccation and cryopreservation on the cellular viability of isolated embryonic axes of Litchi. We also suggest that attention should be given to all critical stages of the cryopreservation protocol since minor modifications like slow cooling; slow thawing can improve the rate of survival in Litchi. Also, the present study provides valuable information in developing appropriate conservation protocols for germplasm diversity of recalcitrant species such as Litchi.

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