VIABILITY OF CRYOPRESERVED EXCISED-EMBRYOS OF
CUCUMIS MELO L

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This project is submitted in partial fulfilment of the requirements for the degree of
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DECLARATION

No portion of the work referred to in this dissertation has been submitted in support of an application for another degree of qualification of this or any other university or institution of higher learning.

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Viability of cryopreserved excised-embryos of Cucumis melo L.

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ABSTRACT

This study was carried out to assess the viability of cryopreserved excised-embryos of Cucumis melo L. that treated using dehydration and vitrification techniques. Embryos sources were high quality, where initial moisture content and germination of C. melo were 67.1% and 76% respectively, and viability of the embryos was 90% when treated using 1.0 M of tetrazolium for 90 minutes. Vitrification and three dehydration techniques were used, and compared, with the highest viability of C. melo was used for storage in liquid nitrogen (-196°C). The highest percentage of viability was obtained from dehydration of excised-embryos under laminar air flow for 6 hours and by using silica gel that gave 81% respectively, and by exposing excised-embryos in 0.4 M sucrose solutions for 60 minutes gave 76% of viability. Beside that, excised embryos that treated through vitrification gave the highest viability at 72%, when exposed to 0.4M of sucrose with PVS2 solutions for 60 minutes. Regression analysis predicted that C. melo embryos could be stored for 3 months, 3 weeks, 3 days if treated with vitrification. Storage for embryos treated with silica gel was 3 months, 2 weeks, 6 days while sucrose was 3 months, 1 week, 2 days. Under laminar air flow was 2 months, 3 week, 6 days, indicating that excised-embryos of C. melo survived better when treated vitrification for long period of storage.

Keywords: Cucumis melo L., cryopreservation, dehydration, vitrification, viability

ABSTRAK

Kajian telah dilakukan untuk mengkaji kebolehidupan embrio C. melo L. yang diawetkan setelah dirawat dengan teknik dehidrasi dan vitrifikasi. Sumber embrio adalah berkualiti tinggi di mana peratus kelembapan dan persembahan peranan yang diperoleh ialah 67.1% dan 76%. Ujian persembahan kebolehidupan adalah sebanyak 90% apabila dirawat dengan 1.0 M tetrazolium selama 90 minit. Vitrifikasi dan tiga teknik dehidrasi digunakan, dan ditandingkan, dengan peratus kebolehidupan C. melo yang terbaik digunakan untuk penyimpanan dalam cecair nitrogen (-196°C). Peratus persembahan terbaik diperoleh dari dehidrasi di bawah aliran udara laminar selama 6 jam dan dehidrasi dengan silika gel selama 24 jam, iaitu sebanyak 81% dikenali dengan mededahkan embrio dalam sukrosa berkepekanan 0.4 M selama 60 minit iaitu sebanyak 76%. Selain itu, embrio yang dirawat melalui vitrifikasi memberikan peratus kebolehidupan terbaik iaitu sebanyak 72% apabila didehidrasi dalam larutan sukrosa berkepekanan 0.4 M dengan PVS2 selama 60 minit. Analisis regresi menunjukkan embrio dapat disimpan selama 3 bulan 3 minggu 3 hari jika dirawat melalui vitrifikasi. Tempoh penyimpanan embrio yang dirawat dengan silika gel adalah 3 bulan, 2 minggu 6 hari, manakala dengan sukrosa adalah 3 bulan 1 minggu 2 hari. Di bawah aliran udara laminar adalah 2 bulan 3 minggu 6 hari dan memunjukkan bahawa embrio C. melo lebih berpaya aktif hidup jika dirawat dengan vitrifikasi untuk penyimpanan jangka masa yang panjang.

Kata kunci: Cucumis melo L., kriowetan, dehidrasi, vitrifikasi, kebolehidupan
CHAPTER 1

INTRODUCTION

*Cucumis melo* L. is a Cucurbitaceae family, which is also known by their common name as melon. This species is a very variable and has long been cultivated for its edible fruit. As a result, a number of distinct forms have arisen and there are many named varieties within each of these forms (Uphof, 1959; Facciola, 1990). One of the most popular melons is the honeydew melon that belongs to the Inodorus group of *C. melo*, which also includes the casaba melon. Honeydew is an American name for the French variety 'White Antibes;' which was grown for many years in southern France and Algeria for foreign shipment (Stephens, 1994).

The honeydew plant is similar to cantaloupe except for more lobbing of the leaf, the fruits are distinctive. They are round to slightly oval, about 8 inches long, and are extremely smooth with no netting or ribs. Some soft hairs are present on the surface in early stages. Rind colour is greenish white when immature, becoming somewhat creamy yellow when ripe. The flesh is light green, thick, juicy, sweet, and uniquely flavoured (Stephens, 1994). They grow in tropical and temperate subtropical places, where the conditions are humid and hot (Mills, 2001). Moreover, the plant also prefers light (sandy), medium (loamy) and heavy (clay) soils and requires well-drained soil. It cannot grow in the shade. Because of its climbing nature, *C. melo* is also known as vine crop. It can grow to 1.5m high (Anonymous, 1997).

It flowers from July to September, and the seeds ripen from August to October. The flowers are monoecious, meaning that the flower is an individual flower, which are either male or female, but both sexes are found on the same plant. Then, insects will pollinate the flower and the plant is
self-fertile (Anonymous, 1997). The fruit is very watery but with a delicate flavour and, it is very refreshing. It rich with vitamins B and C. The flesh of the fruit can be dried, ground into a powder and used with cereals when making bread and biscuits. The size of the fruit varies widely between cultivars but is up to 10cm long and 7cm wide (Huxley, 1992).

The seed is rich in oil with a nutty flavour but very fiddly to use because the seed is small and covered with a fibrous coat. The seed contains between 12.5 - 39.1% oil. Edible oil is obtained from the seed (Tanaka, 1976; Facciola, 1990). The honeydew fruit that include in C. melo varieties inodorous group is propagated by seeds. The seeds can be planted either by direct seeding or through transplant. Transplant is more favourable because it used less seeds compared to direct seedlings and it produce fruits earlier. Direct-seeding works best in temperature that range from 23 –24°C, but the acceptable germination range is from 20 - 45°C (Mills, 2001).

Cryopreservation is a technique that is used to store germplasm for a long period of time without changing the viability of the germplasm. It refers to the placing and holding of biological materials at low temperatures in a manner such that the viability is retained after thawing. Cryopreservation in liquid nitrogen is the most convenient techniques for long-term storage. At liquid nitrogen temperature (-196°C), all cellular divisions and metabolic events are ceased. The plant material can be stored without alteration or modification for a theoretically unlimited period of time (Towill, 1991).

Cryopreservation is currently being developed by agencies such as Kings Park and Botanic Garden (West Perth, Australia) to conserve threatened species. The advantages of cryostorage
over conventional forms of germplasm storage include: reduction in maintenance costs associated with living collections; reduction in storage space; minimization of losses due to diseases and contamination (with living collections); and minimization of somaclonal variation (Bajaj, 1995).

Cryopreservation of embryos, embryonic axes prove to be useful tool for the conservation and for the establishment of germplasm banks of recalcitrant, intermediate and rare plants that are threatened with extinction. The used of zygotic embryos or embryonic axes is much advocated for rescue of hybrid intergeneric crosses, transformation studies, genetic resources conservation and they need less space for storage. These embryonic structures have been widely used for cryogenic storage especially for recalcitrant and sub orthodox seed species during the last decade (Engelmann, 1995). Where feasible, cryopreservation is currently the only safe and cost-effective methods for long-term conservation of the germplasm of species that are vegetative propagated or has seeds that are recalcitrant to storage (Pence, 1995).

Cryopreservation of embryogenic cultures could be useful for reliable long-term maintenance of genetic resources, and therefore for alleviating the effects of tissue-culture-induced somaclonal variation, for enabling efficient recovery of propagules and for reducing labour and supply costs. The combination of somatic embryogenesis and cryopreservation provides a powerful tool to considerably improve the ability to select superior genotypes in tree species. Thus, embryogenic cultures could be maintained through cryopreservation for several years while the best genotypes are selected as determined from long-term field trials (Vendrame et al., 2001).
1.1 Problem Statement

*C. melo* cultivation and population is becoming of great concern in particular the aspect of its protection and conservation. The increase in the demand of *C. melo* for economic purposes and the difficulties of conserving the germplasm (seeds and embryos) of this *C. melo* due to lack of research being carried out, influenced many researchers to investigate ways how to conserve this *C. melo* germplasms for long period, without injuring the embryos. The humid tropical conditions associated with diseases and insect problems have subjected *C. melo* as a poor choice for both gardeners and commercial farmers. The germplasm of many strains or varieties available are extremely susceptible to long-term storage. Perhaps *C. melo* crops will face increase extinction problem if there is no legalization on how to maintain and conserve the germplasm (seeds and embryos) in a long-term storage, because the crops is solely cultivated using seeds. Only high quality seeds with vigorous embryos can be stored for a long period before used in cultivation of *C. melo*.

1.2 Objectives

The main objectives of this study are:

1. To assess the suitability of cryopreservation of excised-embryos of *C. melo*.
2. To evaluate the effectiveness of dehydration and vitrification techniques in preserving excised-embryos of *C. melo*.
3. To determine which technique of cryopreservation is most suitable for preserving *C. melo* excised-embryos for long term-storage.
CHAPTER 2
LITERATURE REVIEW

2.1 Inodorous (honeydew).

Honeydew melon belongs to the *Cucumis melo* L. *Inodorous* group that includes crenshaw, casaba and other mixed melons. Honeydews are harvested by maturity and not by size. Maturity is difficult to judge because no clear abscission (slip, separation) from the vine occurs. Maturity classes are grouped predominantly by changes in ‘ground colour’ from greenish to cream with yellow accents. There are 3 types of commercial maturity classes as listed below:

1. **Mature, Unripe.** Ground colour white with greenish accents, no characteristic aroma, peel fuzzy/hairy and not waxy. California Grade Standards establish a minimum legal harvest index of 10% soluble solids.

2. **Mature, Ripening.** Ground colour white with slightly discernible green tint, slightly waxy peels, blossom-end firm and unyielding, no or slight aroma. Preferred commercial maturity class.

3. **Mature, Full Ripe.** Ground colour creamy white with yellow accents, clearly waxy peels, characteristic aroma noticeable, and blossom-end yields slightly to press.

This honeydew needs about 85-90% humidity. High relative humidity is essential to prevent desiccation and loss of glossiness. However, extended periods of higher humidity or condensation may encourage the growth of surface molds (Trevor et al., 2002). They don’t have a very long shelf life; therefore varieties have been bred with reasonably robust rinds to handle
long distant transit. The variety and complexity of flavours, sizes, flesh colours and textures makes the melon one of the most exciting and interesting fruits there is. It is also an important source of some nutrients. High in Potassium, *C. melo* varieties rock melons/cantaloupes are an excellent source of vitamin A. They are the second best source (after mangoes) of all the fruit with a very respectable 3,224 International Units per 100 g. In addition, normal serving are similar to half daily vitamin C requirements for adult, making them a very good vitamin C source (Wang *et al.*, 1996; Joseph *et al.*, 1999; Vinson *et al.*, 1998).

2.2 Cryopreservation

The historical roots of plant cryopreservation lie partly in studies of cold-hardiness and freezing injury in whole-plant material (Levitt, 1966; Li and Sakai, 1978). The ability to improve plant tissues after exposure to ultra-low temperatures such as that of liquid nitrogen (-196°C) was recognized by Sun in 1958. Comprehensive subsequent studies by Sakai and colleagues (Sakai, 1969; Sakai and Yoshida, 1967; Sakai *et al.*, 1968) explored mechanisms of freezing injury and determined interaction between freezing and thawing rates.

In fact, the effect of low temperatures on plant and animal cells has been studied over the last 100 years, due to the interest in producing frost-resistant plants (Querol, 1987). However, it was not until 1973 that Nag and Street proposed true cryopreservation in liquid nitrogen for cells of *Daucus carota* and by Sakai and Sugawara (1973) for callus of *Populus euphratica*. These early studies drew upon the knowledge of workers in the medical and microbiological fields,
notably in the areas of freezing requirements and the application of cryoprotectant chemicals applicable to other biological systems.

However, later studies have demonstrated that, outside the broad biophysical and biochemical generalizations, higher plant systems require careful examination to match cryopreservation procedures to morphology. Furthermore, in plant tissue culture, higher standards of survival in terms of viable densities, and also arguably, stability of genotype and phenotype are required. The above realities have been evident in the achievements of the decade following the landmark reports of Nag and Street (1978) and Sakai and Sugawara (1978).

Attention has turned increasingly to emergent preservation protocols, which offer high rates of survival and recovery of cultures that resemble unfrozen controls in all measurable respects. Most recently, qualitative aspects of recovered cultures have established particular awareness, with care being given to their biochemical evaluation and physiological performance. These aspects are of critical importance in the widespread acceptance of cryopreservation as a tool in plant biotechnology (Burnett et al., 1986).

The term cryopreservation is used exclusively to cover the storage of living biological material at ultra-low temperatures. This normally occurs at or near the temperature of liquid nitrogen, -196°C (Withers, 1988). As cited by Yamada et al., (1991), cryopreservation may be useful method for a long-term storage using a minimum of space and maintenance. This is a promising technique that can be applied in storage, as it is capable of retaining the gene pool of plant. The
basic concept of liquid nitrogen preservation is that at this temperature, all metabolic processes in seeds are essentially stopped and held in suspended animation (Krishnapillay et al., 1994).

For cryopreservation of endangered species, shoot apices are the preferred tissue source as they allow for the conservation of genetically stable tissues, which are essential for future reintroduction programs. Furthermore, cells found in the apical region of shoot apices are much more amenable to cryostorage protocols as they consist of small thin-walled actively dividing cells that have small vacuoles and a high nucleo-cytoplasmic ratio (Engelmann, 2000). For embryos, cryopreservation is more critical compared to other parts of a plant. The dry and freezing condition can cause injury to the embryo and this can lower the percentage of viability.

The cryopreservation treatment of embryo is often done to plants with big and hard seeds. This kind of embryo can tolerate a low moisture level that is 12 – 13% (Robert, 1975). According to Withers (1988), a typical preservation procedure consists of the following stages: pre-growth, cryoprotection, freezing, storage, thawing and recovery. These stages are usually applied to the techniques of cryopreservation process such as dehydration, vitrification, encapsulation and slow freezing. These four techniques are usually done before the germplasm are plunged into liquid nitrogen for storage process.

2.3 Cryoprotectant

Very few higher plant tissues can survive exposure to ultra-low temperatures without pre-treatment by cryoprotectant chemicals (Burnett et al., 1986). Cryoprotectant enhances survival of hydrated tissues after exposure to low temperatures (Frinkel et al., 1985). The cryoprotectant
differs greatly by their molecular weight and their structure. The low molecular weight compounds are such as sucrose, glucose, glycerol, praline and mannitol whereas the high molecular weight compounds are polyvinylpyrrolidone (PVP), hydroxyethyl starch (HES) and polyethylene glycol (PEG). The uses of this cryoprotectant are applied to the embryo, nuclear cells, and meristem. Effective cryoprotectant must permeate the cell, and the above three do so fairly rapidly in many plant cells (Towill, 1991). They function by altering certain physical properties of water, such as decreasing the freezing point of water, lowering the rate of ice crystal growth and changing the shape of ice crystals. In addition, they have certain protective properties. Most of the successful mixtures of cryoprotectants have been composed empirically. Cryoprotectants can also be toxic to plant cells, especially when used at higher concentrations or at higher temperatures (Arakawa et al., 1990; Fahy et al., 1990).

2.4 Cryostorage

Certainly the preferred storage condition, if available and affordable, is at cryogenic temperatures, which for the purpose of this article are below about -130°C. This can be achieved by a mechanical refrigeration system, but is more commonly accomplished either in the vapor phase over liquid nitrogen (approx. -160°C) or within the liquid nitrogen (−196°C). The use of such low temperatures should able to minimize deterioration. Liquid nitrogen (−196°C) is routinely used for cryogenic storage, since it is relatively cheap and safe, requires little maintenance and is widely available. Below −120°C the rate of chemical or biophysical reactions is too slow to cause biological deterioration (Kartha, 1985). Only in the long term might there be a small risk of ionizing radiation causing genetic changes in materials stored at cryogenic temperatures (Grout, 1995).
2.5 Viability (TZ) test

The tetrazolium test is widely recognized as an accurate means of estimating embryo viability. Tetrazolium test will give the percentage of viable embryos. Tetrazolium is a biochemical compound (2, 3, 5-triphenyl tetrazolium chloride) that is used for assessing the quality of seeds, embryos or tissues. The main functions of tetrazolium are estimating the viability, assessing vigour and diagnosing physiological problems in particular seeds, embryos or tissues. It is a water-soluble powder, white or light yellow in colour. Generally, concentration of 0.1, 0.5 and 1.0% on w/v basis is used (Agricultural Experiment Station, 1962).

2.6 Dehydration

The dehydration technique that is usually used to dehydrate the water content in the seeds or meristems has been applied to several species of plants in cryopreservation process. Dehydration is conducted using silica gel, desiccating under laminar flow hood and cryoprotective method using the plasmolysis by a progressive concentration of sucrose. A dehydration step, necessary to avoid the formation of intracellular ice crystals, results in concentration of solutes in cells and in strong plasmolysis of cells. Removal of water can lead to 'solute effects' in the cell, such as pH changes, increased electrolyte concentrations and macromolecular interactions (Towill, 1991).

Problems associated with plasmolysis may especially arise when cells have to deplasmolyze after thawing. For example, osmotic contraction can result in irreversible endocytotic vesiculation, which will result in lysis during osmotic expansion, because new membrane material is not rapidly enough available to facilitate deplasmolysis. In addition, dehydration increases the gel-to-liquid crystalline phase transition temperature (Tm) of lipids. As a result, membrane lipids
undergo phase transition and phase separation at higher temperatures (Steponkus, 1984). Study has showed that loss of osmotic responsiveness of isolated protoplasts as a result of osmotic dehydration is associated with lateral phase separations, formation of a particulate lamellae and lamellar-to-hexagonalIII phase transitions. Membranes may even undergo fusion when they are brought into close contact during plasmolysis (Stepokus et al., 1992).

Previous research shows that the somatic embryos were dehydrated slowly by sub-culturing with a progressive increase in concentration. The result indicates that the walnut somatic embryos are particularly tolerant to a high concentration of sucrose. Experiment on the cryopreservation of embryonic axes of almond (Prunus amygdalus Batch) has been successful using this dehydration technique (Chaudry and Chandel, 1995). This technique also successfully applied to somatic embryos of Coffea arabica (coffee), Manihot esculenta (tapioca), Phoenix dactylifera (dates) and Pison sativum (pear) (Mycoc et al., 1995).

2.7 Vitrification

Vitrification is a process that produces a glasslike solidification of living cells that completely avoids ice crystal formation during cooling. Equally important, the vitrification process completely avoids ice crystal formation in cryopreserved cells during warming to recover the cells for biological applications. Retrospectively, the phenomenon of vitrification was first investigated and described at the turn of the 19th century (Tammann et al., 1898).

The founder of cryobiology, Luyet (1937), recognized the potential of achieving an ice-free, structurally arrested state for cryopreservation more than 60 years ago and described it in his
classical studies. Subsequently, it was generally recognized that supporting solutions for vitrification would be better for the preservation of living cells and tissues than would solutions that crystallize and hence damage cells during cooling and warming.

According to Fahy et al., (1984), vitrification refers to solidification ("glassification") of the systems during cooling without ice formation. In 1985, a new method for cryopreservation of animal cells was reported (Rall and Fahy, 1985). This so-called vitrification procedure is based on severe dehydration at non-freezing temperatures by direct exposure to concentrated cryoprotectants (total concentration ranging from 5-8 M), followed by rapid freezing. The rapid cooling rates prevent nucleation and growth of ice crystals and facilitate vitrification of the surrounding medium as well as cell contents. After storage, rapid warming rates are used to prevent devitrification during thawing.

The first successful vitrification procedures for plant cell suspensions were developed in 1989 (Uragami et al., 1989). The main advantage of the vitrification procedure is that an expensive programmable freezer is not required. Furthermore, vitrification uses ultra-rapid cooling rates. Therefore tolerance to vitrification is primarily a matter of dehydration tolerance (Langis and Steponkus, 1991). Previous research has been reported that cryopreservation protocols in banana was already being developed for seeds, zygotic embryos (Abdelnour-Esquivel et al., 1992), embryogenic cell suspensions (Panis et al., 1990), proliferating meristem cultures (Panis et al., 1996) and apical meristems (Thinh et al., 1999) excised from rooted in vitro plants.
For the conservation of the diversity in Musaceae, the two latter tissue types deserve preference. Indeed seed and zygotic embryos are only available for seed bearing; non-edible bananas and the induction of embryogenic cell suspensions in banana are inefficient and not possible for all genotypes (Panis and Thinh, 2001; Strosse et al., 2003). Thinh et al., (1999) has been reported that cryopreservation through vitrification also has been applied for in vitro grown shoot-tips of banana.

According to Sakai (2000), cryopreservation of biological tissues can only be successful without the formation of intracellular ice crystals since they cause irreversible damage to cell membranes and thus destroy their semi-permeability. Crystal formation without an extreme reduction of cellular water can be prevented by vitrification, i.e. the non-crystalline solidification of water. Two requirements must be met for a solution to vitrify: (i) rapid freezing rates (normally by plunging explants enclosed in a cryotube into liquid nitrogen); and (ii) a concentrated cellular solution. The latter is obtained through air-drying; freeze dehydration, application of penetrating or non-penetrating substances or acclimation (Panis et al., 2004).
CHAPTER 3
MATERIALS AND METHOD

3.1 Material

Fresh and matured fruits of *C. melo* were obtained from the local market in Kuching, Sarawak. Seeds were extracted from the fruits, cleaned and sterilized by soaking in 70% w/v alcohol solution for 5 minutes. After that, the seeds were rinsed three times with distilled water and dried under laminar airflow hood for 2 hours to remove excess water from the surface of the seeds. The selected seeds were dried again and dusted with Ceptan 50 WP to avoid contamination by fungi. Seeds were kept in airtight container and this container was placed in desiccators in air-conditioned room.

Embryos were extracted from the seeds for treatments in the experiment. The seeds were soaked in distilled water for 1 hour to avoid them from drying and make it easier to remove the coats. The seeds were bisected laterally and seed coats were removed using sharp scalped and forceps. Cotyledons were opened then embryos were taken out and placed on a moistened filter paper in petri dish. All the works were done under 'laminar flow' condition and aseptic environment to avoid the growth of microorganisms that can contaminate and destroy the embryos.
3.2 Method

3.2.1 Preliminary Evaluation

Moisture content, viability and germination tests were carried out to evaluate the viability of the seeds prior to extracting the embryos for use in the subsequent experiments. This was conducted to ensure that the seed lot used was of high quality.

3.2.2 Moisture Content Test

Four replications of each contained 25 embryos (4x 25 embryos) were placed in aluminium foil saucers and weighed using an electrical scale. The aluminium foil saucers were then put in an oven for 48 hours at 60°C and reweighed after removed from the oven. The moisture content percentage was calculated using the formula recommended by the International Seed Testing Association (ISTA, 1976):

\[
\text{Moisture Content (\%)} = \frac{(b) - (c)}{(b) - (a)} \times 100\%
\]

where:

(a) = weight of empty aluminium foil saucer

(b) = weight of (a) + embryos before drying in the oven

(c) = weight of (a) + embryos after drying in the oven
3.2.3 Viability (TZ) Test

The embryos were sorted into four replications that contained 25 embryos each. This was carried out to determine the suitable tetrazolium concentration for the embryos. Concentrations of tetrazolium that used were as follows; 0.1, 0.5 and 1.0%. The embryos were placed in beakers for each replicates and wrapped with aluminium foil. Then tetrazolium solution was poured in each beaker and incubated in incubator at 35°C for the periods of 0, 30, 60, 90, 120, 150, 180 and 240 minutes. After each period, tetrazolium solution was removed and rinsed with distilled water for 3 to 5 times. The colour of stained embryos and the degree of staining was observed. The red stained embryos were considered viable. The best treatment in tetrazolium tests was used as standard for evaluating the viability of embryos in after vitrification and dehydration treatments.

3.2.4 Germination Test

The Standard Germination Test was carried out according to the Rules for Seed Testing (AOSA, 1985). Four replications that contained 25 embryos each were germinated on an MS (Murashige and Skoog) medium. Petri dishes contained the MS medium were wrapped with parafilm to avoid contamination. The petri dishes were incubated in ‘Plant Growth Chamber’ at 29°C. Germination was obtained within 3 to 5 days. Germination percentage was calculated using this formula;

\[
\text{Germination (\%)} = \frac{\text{Total of germinated embryos}}{\text{Total of embryos}} \times 100\% 
\]