Optimization of Sterilizing Protocol on *In Vitro* Germination of *Solanum melongena* L. cv. Mini Cluster

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Optimization of Sterilizing Protocol on *in vitro* Germination of *Solanum melongena* L. cv. Mini Cluster

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A thesis submitted in partial fulfilment of the Requirement of The Degree Bachelor of Science with Honours
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Programme of Resource Biotechnology
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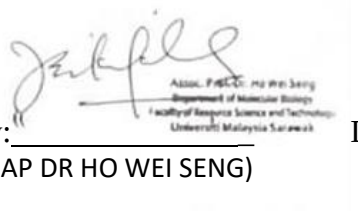

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ABSTRACT

In plant tissue culture, an optimum sterilizing protocol is important and crucial to obtain the best result without any contamination. Contamination during *in vitro* germination may affect our results since the contaminants will compete for nutrients which may lead to insufficient nutrients for the seeds to grow. In this study, the seeds of *S. melongena* L. cv. mini cluster is used for *in vitro* seed germination. Although many studies have been conducted on finding the optimum sterilizing protocol for eggplant seeds, but only a few research that focuses on this specific cultivar, which is *S. melongena* L. cv. mini cluster. Hence, this study focuses on sterilizing the seeds of *S. melongena* L. cv. mini cluster with different concentrations of Clorox at different duration to find the optimum sterilizing protocol. The seeds were treated with 15%, 35% and 50% Clorox for 10 minutes, 15 minutes and 20 minutes respectively. After 6 weeks of germination and observation, it is found that seeds sterilized with 70% ethanol and 50% Clorox for 15 minutes have 0% contamination and 100% germination. Despite this research is only focusing on mini cluster cultivar, however other sterilizing protocol still can be used in sterilizing this cultivar and this research can be used on other cultivars as well.

Keywords: surface sterilization, *in vitro* germination, contamination, *Solanum melongena* L. cv. mini cluster

A**BSTRAK**

Di dalam kultur tisu tumbuhan, protokol penstrilan yang optimum adalah sangat penting dalam memperolehi keputusan yang terbaik tanpa sebarang kontaminasi. Kontaminasi semasa pencambahan in vitro boleh menganggu keputusan akhir kerana kontaminan akan bersaing untuk mendapatkan nutrisi di mana biji benih akan memperolehi nutrisi yang kurang untuk tumbuh. Di dalam kajian ini, biji benih S. melongena L. cv. terung mini akan digunakan untuk pencambahan in vitro. Walaupun terdapat banyak kajian yang dilakukan untuk mencari protokol penstrilan yang optimum untuk biji terung, namun hanya sedikit kajian yang telah dibuat ke atas kultivar terung mini. Oleh itu, kajian ini berfokuskan kepada penstrilan biji S. melongena L. cv. terung mini menggunakan kepekatan Clorox yang berbeza pada masa berbeza untuk menentukan protokol penstrilan yang optimum. Biji benih tersebut dirawati dengan 15%, 35% dan 50% Clorox selama 10 minit, 15 minit dan 20 minit masing-masing. Selepas 6 minggu pencambahan dan pemerhatian, telah didapati bahawa penstrilan dengan 70% ethanol dan 50% Clorox selama 15 minit mempunyai 0% kontaminasi dan 100% germinasi. Walaupun kajian ini hanya memfokuskan kepada kultivar terung mini, namun protokol penstilan yang lain masih boleh digunakan ke atas kultivar ini dan kajian ini juga boleh dilakukan ke atas kultivar lain juga.

***Kata kunci**: pensterilan permukaan, pencambahan in vitro, kontaminasi, Solanum melongena L. cv. terung mini*

TABLE OF CONTENT

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

B5	Gamborg
CDC	Center for Disease Control and Prevention
cv.	Cultivar
HCl	Hydrochloric acid
MS	Murashige and Skoog
NaOCL	Sodium hypochlorite
NaOH	Sodium hydroxide

CHAPTER 1

INTRODUCTION

Solanum melongena L., or also known as eggplant, is the third most essential crop in the family *Solanacae* after tomato and potato (Ali & Tsou, 1997). Eggplant has been considered as the most influential vegetables due to its delicate taste, a good source of fibers, vitamins and minerals as well as antioxidant activities that are exhibited by polyphenols which can be found in eggplants (Nisha et al., 2009). Furthermore, it has been proven that the eggplant contains low calorie and fat content which can assists in weight loss and prevent the risks of cardiorespiratory illness (Robinson & Saranya, 2013). Previous studies also suggests that the phenolic compounds in eggplant for instance, chlorogenic acid, may help in prevention of certain diseases such as bronchitis, asthma, diabetes, and arthritis (Pratap et al., 2011; Scalzo et al., 2016). This beneficial trait drives researchers to focuses on wild relatives of eggplants through biotechnological, genomics and conventional approaches (Gramazio, 2018).

Conventional plant breeding and biotechnological methods had been used in eggplant breeding in every scientific research (Plazas et al., 2016). *In vitro* germination is one of the procedures for eggplant breeding. Eggplants are often bred due to yielding, tolerance to biotic and abiotic stress, and improvement of quality (Alam & Salimullah, 2021). Nonetheless, inadequate amount of resistance of abiotic and biotic stress are often found in cultivated eggplant genotypes whereas crossing of eggplants with its wild types often gives negative result because of sexual incompatibilities (Alam & Salimullah, 2021; Sękara et al., 2007).

In a successful plant breeding, the source of explants plays a major role as explants that are obtained from the field have high chances of culture contamination (Kaur et al., 2014). There are a few factors that may lead to successful germination. For instance, moisture, light, temperature and more (Kaur et al., 2014). Surface seed sterilization plays an important role in overcoming the problem of contamination of the seeds, eventually causing a successful germination rate. Primary inoculum is often due to seed-borne infections and this problem should be solved by treating the seeds before sowing which may lead to a successful germination and healthy crop (Habib et al., 2007).

Before any *in vitro* germination can be done, the seeds or the explants must first be surface sterilized. In plant tissue culture, surface sterilization is a crucial step as high concentration of contaminants may have negative effects in cell division which leads to growth and development restriction (Bhadane & Patil, 2016). There are a lot of factors that influenced a successful *in vitro* germination such as competent explants, plant material and most importantly the sterilization protocol (Rezadost et al., 2013).

Despite much research on *in vitro* germination has been done, but there is less research that focuses on the optimum sterilizing protocol to be used. It is very important to know that the concentration of the sterilization factor used must be the optimum to avoid contamination or phytotoxicity. Phytotoxicity can delay the seed germination rate or inhibit the plant growth (Purcell, 2009). Thus, in this study, the optimum concentration and time of the sterilizing agent used must be determined empirically to avoid such consequences from happening. Different cultivars have different optimum sterilizing protocol. There are only a small amount of research that focuses on this specific eggplant cultivar which is mini cluster. Other than that, most study suggest having surface sterilization with mercury chloride. Mercury chloride is known to be very toxic and dangerous to be used as sterilizing agent (Barampuram et al., 2014). Therefore, this research will be focussing on sterilization of

Solanum melongena L. cv. mini cluster with other sterilizing agent, specifically sodium hypochlorite which can be found in Clorox. In this research, the effect of different concentrations of Clorox on eggplant seeds, specifically 15%, 35%, and 50% at different duration, at 10 minutes, 15 minutes and 20 minutes respectively will be studied. Hence the main objective of this research is to determine the optimum sterilizing protocol to sterilize the seeds of *Solanum melongena* L. cv. mini cluster.

CHAPTER 2

LITERATURE REVIEW

2.1 Selection of species studied

2.1.1 Family Solanaceae

The family Solanaceae is a huge plant family that consists of more than 3000 species such as potato, tomato, pepper, and eggplant (Wu & Tanksley, 2010). This family is high in diversity as it ranges from perennial trees to herbaceous annual species which has a large area of habitats from deserts to rainforests (Knapp et al., 2004). What makes the family Solanaceae unique is due to its importance in terms of flortistic, phylochemistry, economic and ecobiological (Ahmed et al., 2019). Although it has a plethora of diversity, only a few members of the family Solanaceae that gives positive impact towards the human civilization that acts as food sources, ornamental and drugs (Gebhardt, 2016). For instance, potato and eggplant as food sources, petunia and Detunia as ornamental, and tobacco and Atropa ad drugs (Gebhardt, 2016). Hugo de Vries and Karl Correns rediscover the pioneer work of Gregor Mendel in the beginning of the twentieth century where seven genera species of the family Solanaceae have become a part of the genetic research that act as model plants due to their importance as crops (Gebhardt, 2016). In Figure 2.1, it shows the different variety of the family Solanaceae (Ara et al., 2011). In Table 2.1, there are eight most essential genera in this family and the largest genera is Solanum (Knapp, 2004).

Figure 2.1 Plant varieties of the family Soalanceae; A. *Solanum nigrum* L., B. *Solanum spirale* Roxb., C. *Solanum indicum* L., D. *Solanum xanthocarpun* Schrad & Wendl., E. *Solanum torvum* Swartz., F. *Physalis minima* L., G. *Nicotiana plumbaginifolia* Viv., H. *Datura metel* L. (Ara et al., 2011).

Table 2.1 Largest genera of the family Solanaceae in rank order (Knapp, 2002; Wikipedia)

Genera	Approximate Number of Species
Solanum	1,330
Lycianthes	200
Cestrum	150
Nolana	89
Physalis	85
Lycium	85
Nicotiana	76
Brunfelsia	45

2.1.2 Genus *Solanum*

The genus *Solanum* is known as one of the largest genera in angiosperms and also plays an important role in the family Solanaceae (Kaunda & Zhang, 2019). This genus contains about 2000 species which is scattered among subtropical and tropical regions in Asia, non- arid Africa, America, Australia, India as well as Brazil (Yokose et al., 2004). This genus is an accepted species that can be found globally in most tropical and temperate biome with habitats ranging from high elevation grasslands to deserts to rainforests (Echeverría-Londoño et al., 2020). This genus gives a huge impact towards the economy on a global scale, for instance beneficial in terms of agriculture (e.g., *S. tubersoum* L., potato; *S. melongena* L., eggplant), important local fruits crops (e.g., *S. muricatum* Aiton, pepino; *S. quitoense* Lam., naranjilla) as well as medicinal and herbaceous plants (e.g., *S. marginatum* L.f.,S. *aviculare* Aiton) (Echeverría-Londoño et al., 2020).

2.1.3 *Solanum melongena* L.

Based on Beentje (2010), *Solanum melongena* L, commonly named aubergine or eggplant comes from the family Solanaceae which is also the family name for potato, tomato, nicotine and the nightshade plant which is very poisonous and deadly. The Latin name *melongena* comes from the Italian word ‘melanzane’ which translates to ‘mela insane’ or mad apple (Beentje, 2010). Eggplants are typically grown as an annual and displays a spiny and bushy stem, large, ovate and slightly lobed leaves, flowers which are pendant violet, as well as an egg-shaped berry fruit which has a shiny surface that has a plethora of colors ranging from dark purple to white (Britannica, 2019). Eggplants are robustly andromonoecious, a developed breeding system manifested by a single or a cluster of large hermaphroditic

flowers at the foot of the inflorescence, with more distal short blooms primarily containing the male component (Whalen and Costich, 1986). Nowadays, most varieties of eggplants have been chosen to bear a single long flower to have a better fruit size consistency due to multi-fruited inflorescence might contribute to different fruit sizes (Knapp et al., 2013). However, eggplants is least recognized as a target for molecular research due to most of the agronomical traits in eggplant can be found in other solanaceous crops like tomato or potato (Wu et al., 2009). Nevertheless, eggplant has a special aspect from botanical and agronomical point of view compared to tomato and potato where eggplants fruits are bigger in size, low intolerance towards abiotic and biotic stress as well as parthenocarpy without negative pleiotropic effects (Saito et al., 2009). Furthermore, from the point of view of food research, eggplants have recently reconsidered to be a better source of radical scavengers, for instance, anthocyanins and phenolics (Azuma et al., 2008).

2.2 *In vitro* germination

In vitro germination is one of the biotechnological approaches in propagating and conserving plant species that is threatened to keep the variability of plant genetics (Utami and Hariyanto, 2019). It is a biological process that has more than one variable which can be determined by genotypes and physical factors, for instance, type of plant growth regulators, concentration of plant growth regulators, source of carbon, medium composition, disinfection and sterilization procedures, pH, temperature, and also light intensity (Genze et al., 2020; Hesami and Jones, 2020). *In vitro* germination is crucial in order to prover an increase in multiplication rates from tissue segments at the same time, it is an adequate tool for obtaining a huge number of contaminant-free samples (Koné et al., 2015). *In vitro* germination usually uses different type of media depending on the nutrient needs of the seeds or explants. For

example, Murashige and Skoog (MS) media and Gamborg B5 media (Murashige & Skoog, 1962; Gamborg et al., 1968)

2.3 Contamination during plant tissue culture

During plant tissue culture contamination often takes place due to inoculation of explants infected by microbes that is resistance to surface sterilization or endophytic which may lead to becoming pathogenic in culture media (Lata et al., 2006). Microbes such as bacteria, viruses and fungi compete with the plant tissue cultures in the media for nutrients (Omamor et al., 2007). This will usually lead to culture mortality, disrupt the plant growth rate, causing tissue necrosis, as well as reducing shoot and root profile (Kane, 2003). Furthermore, poor handling practices, lack of laboratory hygiene, and incompetent sterilization techniques are also one of the reasons how these microbes can contaminate the culture (Fang and Hsu, 2012). In a successful *in vitro* culture, the contamination should not exceed 2% per subculture (Leifert and Woodward, 1997).

2.4 Surface seed sterilization

2.4.1 Ethanol

Ethanol or also known as ethyl alcohol is one of the most used disinfectant and sterilizing agent. Ethanol or alcohol in general helps in antimicrobial action by the mechanism of denaturation of proteins (Centers for Disease Control and Prevention, 2016). Ethanol which is a dehydrating agent is less bactericidal than a mixture of alcohol and water due to denaturing of proteins is way quicker in the existence of water (Block, 2001). Based on a research made by Sykes (1939), alcohol can destroy the dehydrogenases of *E.coli* whereas

Dagley et al. (1959) stated that ethanol can increase the lag phase of *Enterobacter aerogenes* which can be reversed by the addition of amino acids. Ethanol is a strong sterilizing agent but also highly phyto-toxic which means explants should be exposed and treated with ethanol for only a few seconds or a minute (Oyebanji et al., 2009). Water which is a notable content in a denatured ethanol act as a catalyst that helps to slow down evaporation eventually increasing the surface contact time (Origin BG OOD, 2020). 70% ethanol is an adequate concentration to be used for destroying the tissues of microbes such as *Blastomyces dermatitidis*, *Histoplasma capsulatum*, *Cryptococcus neoformans*, and *Coccidioides immitis* (Centers for Disease Control and Prevention, 2016). According to Tiwari et al. (2018), they stated that 70% ethanol is crucial for bacterial disinfection during seed sterilization. Furthermore, 70% ethanol that also contains 30% water is less flammable which decreases the formation of unstable peroxides that may cause combustion when in contact with aluminium (Origin BG OOD, 2020).

2.4.2 Sodium hypochlorite

Sodium hypochlorite, NaOCL, is widely used as disinfectant since the mid-18th century (Miché and Balandreau, 2001). This chemical is a very efficient killer to eliminate bacteria even though with a small micromolar concentrations (Oyebanji et al., 2009). Sodium hypochlorite is an easy to access chemical as it is usually purchased as commercial bleach which makes it the best choice for a sterilizing agent and can easily be diluted to proper concentrations. Nakagawara et al. (1998) stated that sodium hypochlorite is very efficient to kill different types of bacterial strain. According to Rick and Borgnino (1989), for 30 minutes, the implementation of 2.7% sodium hypochlorite, NaOCl, which is the half-strength standard household bleach is good for seed germination. This is due to the factor of

bleach is readily available and it contains 4% NaOCl, 1% sodium hydroxide (NaOH), and 1% amine oxide which can be used as eggplant seeds sterilization (Kaur et al., 2014). Sodium hypochlorite has been proven in fungal disinfection (Tiwari et al., 2018).

2.4.3 Optimization of Sterilizing Protocol

Optimum seed sterilization is important in ensuring a balance of high germination rate, small contamination rate as well as a good and healthy plant growth (Lindsey III et al., 2017). Lindsey III et al. (2017) also mentioned that seeds that are obtained from greenhouse or growth chamber are usually inhabited by various types of microbes ranging from fungi to bacteria. In a study conducted by Foo et al. (2018), the eggplant seeds were surface sterilized with 70% ethanol for 1 minute followed by sterilization with 50% Clorox for 20 minutes. The results give positive results which help in their sterilization protocol. Another study conducted Zayova et al. (2008) where the eggplant seeds were surface sterilized with 70% ethanol for 1 minute followed by sterilization with 15% Clorox for 10 minutes where it shows positive results for the sterilization protocol. Hence, in this study the minimum concentration used for Clorox is 15% for 10 minutes while the maximum concentration is 50% for 20 minutes as shown in Table 2.4.

Table 2.4 Concentration of Clorox and time taken for treatment with different concentration of Clorox

Concentration of Clorox (%)	**Time Taken for Treatment with Clorox (min)**
15%	10
	15
	20
20%	10
	15
	20
50%	10
	15
	20

CHAPTER 3

MATERIALS AND METHODS

3.1 Plant Materials

The seeds of *Solanum melongena* L. cv. mini cluster was obtained from the nearby local nursery. The brand of the seeds that was used was from Baba Smart Grow.

3.2 Seed Surface Sterilization

Seeds of *Solanum melongena* L. cv. mini cluster was soaked in distilled water for 1 hour as part of the pre-treatment procedures. This is to encourage a better seed germination. 70% ethanol was prepared by diluting 95% ethanol with distilled water empirically. On the other hand, by using empirical method, 15%, 35%, and 50% Clorox was prepared by diluting with distilled water as well. Then, the seeds were distributed in 1.5mL tubes with 3 seeds in each tube. Then the seeds in each tube were washed with different concentrations of Clorox, (15%, 35% and 50%) at different duration (10 minutes, 15 minutes, and 20 minutes) respectively. Then, each seed was washed with distilled water three times to remove excess Clorox on the seeds. Then the seeds were washed with 70% ethanol for 1 minute then washed with distilled water again for three times to remove excess ethanol. Then the seeds were dried on dry sterile tissue paper for 3 minutes. Another batch of seeds were also sterilized as replicate.

Another set of experiments were prepared where the seeds were sterilized with different protocol. First set of seeds with 3 replicates were treated with 70% ethanol for 1 minute and

15% Clorox for 10 minutes. Second set of seeds with the same replicates were treated with 70% ethanol for 1 minute only. The third set of seeds were not surface sterilized and germinated on wet filter paper.

3.3 Culture Media

The seeds of Solanum melongena L. cv. mini cluster were cultured on Gamborg B5 medium, supplemented with 20 g sucrose as source of carbon and 8 g agar. The pH was adjusted to 5.6 with either HCl or NaOH. The media then was heated in a microwave and stirred to dissolve the agar. Then the agar was poured onto petri dish and covered to be autoclaved.

3.4 Data Collection

In the experiment, 3 replicates were used for each treatment (3 seeds in one petri dish) and the experiments were repeated twice. The seeds were observed every 3 days and the following data were recorded: percentage of contamination (%) and percentage of seeds germinated (%).