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Sterilization protocol of *Ficus deltoidea* Jack var *kunstleri* (King) Corner

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Abstract: *Ficus deltoidea* has been traditionally used as ahealthcare maintenance and to treat ailments in Malay culture. Due to its scarcity, *in vitro* propagation is seen effective for mass propagation of this species. To achieve successful *in vitro* propagation, sterilization protocol plays a fundamental role particularly if the explants are derived from the field. Therefore, this study was conducted to develop a standardized protocol for sterilization of field-derived explants of *Ficus deltoidea* Jack var *kunstleri* (King) Cornerwhere the type and concentration of sterilants as well as exposure duration were optimized. Preharvest treatment using 2% Benlate and the combination of sterilants such as soaking the explants in 2% Benlate,70% ethanol, 0.1% $HgCl_2$ and 25% commercial bleach plus a few drops of Tween 20resulted in 100% sterile culture.The findingsprovide a groundwork for *in vitro* culture of *Ficus deltoidea* Jack var *kunstleri* (King) Corner.

Key words: *Ficus deltoidea* · Contamination · Leaf · Sterilization · Pre-treatment

INTRODUCTION

*Ficus deltoidea*which is known as 'Mas Cotek' in Malay is a member of mulberry family, Moracea. This plant that serves either medicinal or ornamental purpose is originally found in Southeast Asian countries including the Philiphines, Malaysia and Indonesia [1]. There are seven *F.deltoidea* varieties but *F.deltoidea* Jack var *kunstleri* (King) Corner is the most widely used as ethnomedicine. As for postpartum treatment as it is believed to tighten womb and vaginal muscle [2]. Other traditional uses of this plant is to alleviate diabetes, kidney problem, high blood pressure and diarrhoea [3]. However, despite its wide application it is now facing extinction threat as a result of uncontrolled foraging and deforestation.

*F.deltoidea*is conventionally propagated by seedlings and stem cutting. However, the use of seedlings is not efficient as it reduces viability, germination rate and rooting of the seedlings [4]. For many years, i*n vitro* propagation technique has rapidly replace the conventional propagation in order to increase production of slow-growing plants and to conserve rare and endangered species [5]. As opposed to conventional cutting, microcutting guarantees genetic stability of regenerated plantlets without creating unwanted variations. This characteristics is important in commercial nurseries in order to ensure consistency in quality.

In *in vitro* propagation, rigorous exclusion of contaminating microorganisms is prerequisiteespecially if explants are derived from woody plants [6]. During the long growing period in the fields, plants are continuosly exposed to numerous field contaminants and they may also harbour various insects whose activities further increase the microorganism's contamination in the host plant tissues. Several options are available to complement the usual surface sterilization procedure such as isolation of the mother stock plant from the usual field contaminants and do the pre-treatment of materials in the fieldby spraying fungicide.Fungicide like Benlate, Benomyl and Carbendazim were also used in surface sterilization by dipping the explant in 1% to 10% of the fungicide solution for 10 to 30 minutes [7-9].

In *F. deltoidea*, getting adequate disinfection of the explant material can be of considerable difficulty especially when the explants were found close to the soil. Therefore, a procedure for surface sterilization by determining the type and concentration of sterilants to be used and their exposure time is essential.The choice of an explant can also have a great influence on the success of

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the culture. It is essential to choose an explant with a group of cells that will show the best response when placed in culture. Numerous studies have demonstrated that the type of explant used for *in vitro* regeneration of plant species is determinant of success. As all plant cells have totipotency, leaf, petiole, flower part, root and nodal segment are universally used as an explant in *in vitro* regeneration. The younger leaves especially from stage 1 to 3 counting from the plant's tip are always preferred as an explant as they are still new, young and believed to have less chances of contamination compared to the older stages and percentage of callus induction and fresh weight of callus are higher from younger leaves [10].

MATERIALS AND METHODS

Plant Materials: Leaf and nodal explants of *Ficus deltoidea* Jack var *kunstleri* were collected from a two year-old plant grown at the nursery of Faculty of Bioresource and Food Technology, UniSZA.

Pre-Sterilization of Explants: The leaves from stage one to three were cut from the mother plants at the nursery and placed in ice box to maintain their freshness prior sterilization and inoculation in the laboratory. Collected explants were washed and gently rubbed with a few drops of Teepol and washed under running tap water for about 30 minutes. The explants were immediately transferred into a sterile beaker in laminar air flow cabinet. In the laminar air flow cabinet, the explants were soaked in 70% ethanol (EtOH) for two minutes and rinsed twice with sterile distilled water (dH_2O). Explants were then surface-sterilized either by single or different combinations of sterilants and variable exposure time.

Surface Sterilization with Clorox®: Explants were treated with different concentrations of commercial bleach solution (Clorox®) at different exposure times. The treatments were named at C1-C9 (Table 1). Two drops of Tween 20 was added into each treatment before soaking the explants. The explants were then rinsed three to five times with sterile dH_2O before inoculation.

Surface Sterilization with Mercuric Chloride: The explants were submerged in mercuric chloride ($HgCl_2$) of different concentrations at different exposure times (Table 2). Explants were then soaked in 40% Clorox® for 30 minutes and rinsed five times with sterile dH_2O before being inoculated onto culture medium medium.

Clorox® and Mercuric Chloride Surface Sterilization of *F. deltoidea:* This experiment used combinations of different concentration of Clorox® and $HgCl_2$ (Table 3). Explants were soaked in $HgCl_2$ for five minutes and washed two times with sterile dH_2O. Then, they were soaked in Clorox® for 30 minutes and washed with sterile dH_2O for five times before inoculation onto the medium.

Thiram and Benlate Treatment of *F. deltoidea*: Prior sterilization, the explants were soaked in three different concentrations of Thiram and Benlate (Table 4) for 30 minutes and rinsed until the cloudy colour of fungicide disappeared. These treatments were named as treatment TB1- TB6. Explants were transferred into sterile beaker in the laminar air flow cabinet and soaked in 70% EtOH for two minutes followed by 0.1% $HgCl_2$ for five minutes and 40% Clorox® for 30 minutes for surface sterilization the. The explants were rinsed five times with sterile dH_2O before inoculation.

Table 1: Treatments with Clorox® using various concentrations and exposure times

	Clorox® (%)		
Time (min)	20	40	80
15	C1	C2	C3
30	C4	C5	C6
60	C7	C8	C9

Table 2: Treatments with $HgCl_2$ using different concentration and exposure times

	$HgCl_2$ (%)		
Time (min)	0.1	0.3	0.5
2	M1	M2	M3
5	M4	M5	M6
10	M7	M8	M9

Table 3: Treatments with $HgCl_2$ and Clorox® using different concentration and exposure times

	Clorox® (%)		
$HgCl_2$ (%)	25	50	75
0.05	CM1	CM2	CM3
0.1	CM4	CM5	CM6
0.2	CM7	CM8	CM9

Table 4: Treatments of Thiram and Benlate using different concentrations

Concentration (%)	Thiram	Benlate
1	T1	B1
2	T2	B2
4	T4	B4

Fungicide Pre-Treatment of Mother Plant: The whole mother plant of *F. deltoidea* at nursery was sprayed with 2% Benlate once a week for one month period prior to sterilization and inoculation in the laboratory.

Media Composition and Preparation of Explants: Murashige and Skoog (1962) [11] medium supplemented with 30 g/L sucrose and 1 mg/L 2,4-D was used as the basal media for all experiments. The medium was solidified with 7.5 g/L agar and autoclaved at 121°C for 15 minutes. For inoculation, leaf explants were cut into 1cm x 1cm size along the midrib and incubated at 25±2° C in the dark.

Data Analysis: The experiments were arranged in a completely randomized design (CRD). Observation was made on daily basis in the first few weeks and less regularly afterwards. Data for the number of contaminated, dead and alive explants were recorded. Three replicates of 10 explants for each treatment were used. Results were expressed in mean percentage of fungi and bacteria contamination, dead and alive explants.

Percentage of fungi/bacteria contamination/dead/ alive explants:

$$\frac{Number\ of\ fungi/bacteria\ contamination/dead/alive\ explants}{Number\ of\ total\ explants} \times 100$$

Data were analysed using Analysis of Variance (ANOVA) from Statistical Package for Social Science (SPSS) program. The data were presented as mean ± standard error of mean. Multiple comparisons were done using Duncan's Multiple Range Test (DMRT).

RESULTS

Effects of Clorox® Concentration on Surface Sterilization of *F. Deltoidea*: After 12 days of incubation (DAI), 100% explants from all treatments were damaged by fungus contamination. Infection symptoms were visible at 3 DAI. In general, lower concentration of Clorox® with shorter exposure times caused sooner infection than higher Clorox® concentration and longer exposure times. For example, symptoms of infection could only be seen at 5 DAI in explants treated for 60 minutes (C7-C9) but growth of explants was retarded due to tissue damage. Although none of the explants survived, 40% Clorox® with 30 minutes exposure duration (C5) was selected as the best treatment.

Effects of Mercuric Chloride Concentration on Surface Sterilization of *F. deltoidea*: The use of $HgCl_2$ caused better survival of explants (Figure 1). Fungal contamination was reduced to 64.44% and bacterial contamination was considered low (6.67% to 23.33%). The use of the lowest $HgCl_2$ concentration (0.1 %) at the shortest exposure time (2 minutes) (M1) caused all explants to die. Increasing $HgCl_2$ concentration to 0.3 % (M2) and 0.5% (M3) while maintaining exposure times to 2 minutes was not effective either suggesting that the explants needed longer exposure times. However, this was not the case as the highest concentration (0.5 %) at the longest exposure times (10 minutes) caused some of the explants to die without any contamination symptoms. It was found that 5 minutes treatments (M1-M4) resulted in better plants survival as all treatments at this exposure times produced alive explants. However, since higher percentage of survival (10 %) was shown at 0.1% $HgCl_2$ concentration, M4 was considered as the most effective treatment using $HgCl_2$.

Effects of Clorox® and Mercuric Chloride at Different Concentration on Surface Sterilization of *F. deltoidea:* Although treatment M4 was chosen as the best treatment but the percentage explants survival was very low. Therefore, lower concentration range of $HgCl_2$ and different concentrations of Clorox® were tested (Figure 2). The exposure times for $HgCl_2$ and Clorox® were maintained to 5 minutes and 30 minutes respectively. Regardless of Clorox® concentration, sterilisation with 0.2% $HgCl_2$ were proved toxic to explants as higher number of explants died (CM8 and CM9) although fungal contaminations were significantly reduced as Clorox® concentrations were increased (CM8-CM9). However, decreasing $HgCl_2$ concentration to 0.05% did improved explants survival but resulted in high fungal contamination (82- 87%) regardless of Clorox® concentration. It was found that using 0.1% $HgCl_2$ improved plants survival especially in CM4 where 23% explants were alive. Although bacterial contaminations were significantly reduced when compared to previous experiment, fungal contamination remained a challenge as between 72% to 87% were observed regardless of the sterilants concentration combination.

Effects of Thiram and Benlate Concentration on Surface Sterilization of *F. deltoidea*: Overall, the use of Thiram showed improved sterilization as the occurrence of fungal and bacterial contamination decreased and the number of survived explants increased (Figure 3).

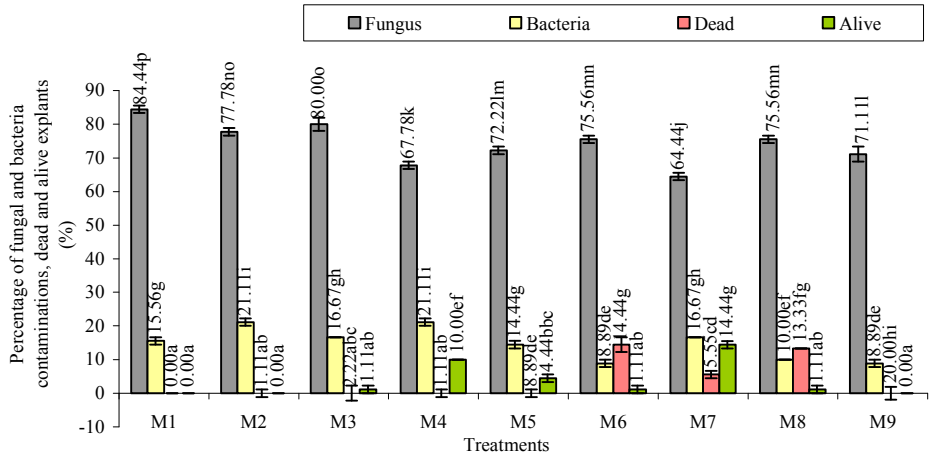

Fig. 1: Surface sterilization of *F. deltoidea* Jack var. *kunstleri* with $HgCl_2$ at varying concentrations at exposure times.

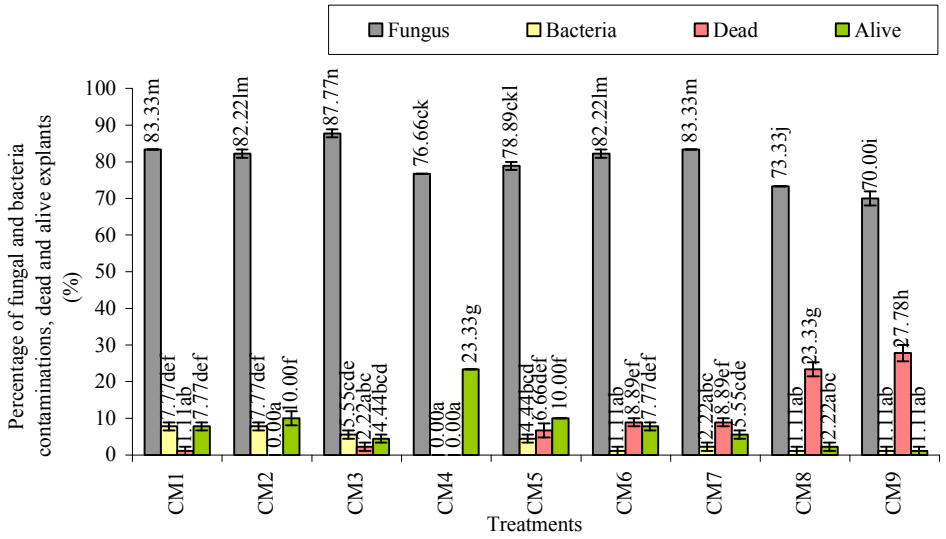

Fig. 2: Surface sterilization of *F. deltoidea* Jack var. *kunstleri* with Clorox®and $HgCl_2$ at varying concentrations at exposure times.

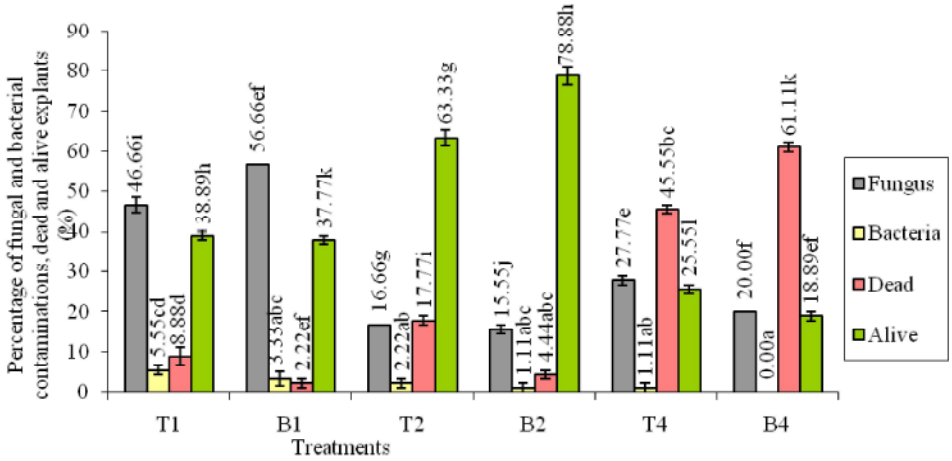

Fig. 3: Thiram and Benlate treatments for pre-sterilization of *F. deltoidea* Jack var. *kunstleri*

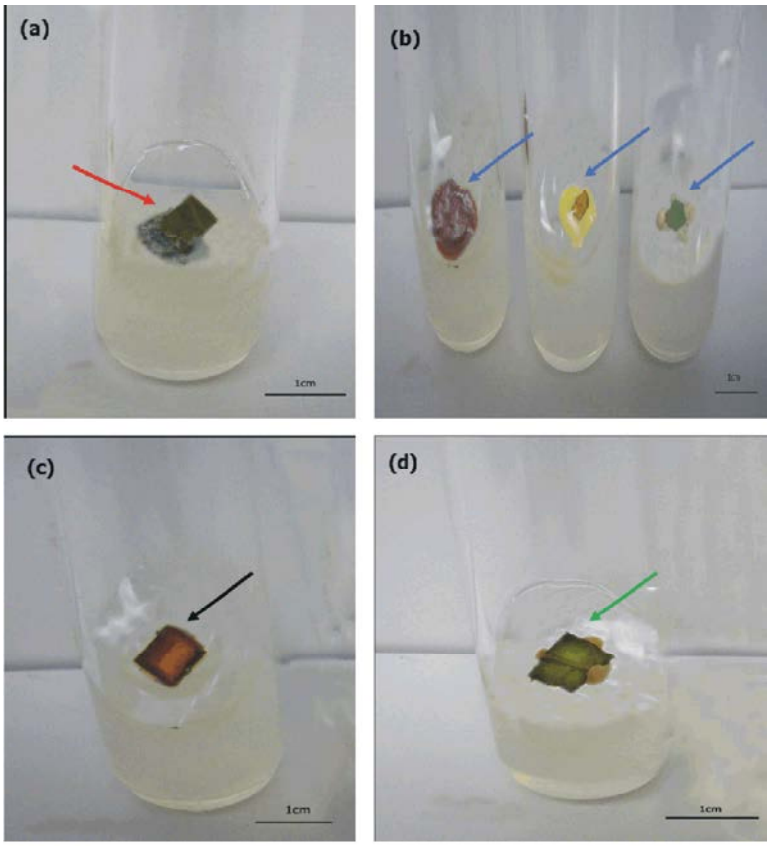

Fig. 4: Field-derived leaf explant of *F. deltoidea* Jack var. *kunstleri.* (a) Fungal contamination (red arrow) after 1 week culture and (b) bacterial contamination (blue arrow) after 3 weeks culture. (c) Dead explant (black arrow) and (d) callusing explant (green arrow) after 4 weeks culture

It could be seen that the fungal contamination were reduced from 46% (TB1) to 16% by using 2% Thiram (TB2). However increasing Thiram concentration to 4% has caused nearly half of the explants died. Excessive Benlate concentration (4%) equally resulted in low explants survival (18%) and high number of cell death (61.11%). Also, Benlate at 2% concentration increased survival rate to 78.88% as compared to 1% Benlate which only showed 37.77% survival rate. Fungal contamination was also reduced nearly four times at this concentration. Because of this, Benlate was considered a better choice than Thiram and the best concentration for it is 2%.

Effects of Fungicide Pretreatment on Mother Plant of *F. deltoidea*: As 2% Benlate was chosen as the best pre-sterilant for *F.deltoidea*, it was then used for pre-treatment of mother plant. In the laboratory, the explants were sterilized by the best treatments from above experiments which is soaking in 2% Benlate for 30 minutes, soakng in 70% EtOH for two minutes followed by 0.1% $HgCl_2$ for five minutes and 25% Clorox® for 30 minutes. The results were compared for the pre-treated and untreated mother plant. Figure 4 clearly shows that the pre-treatment of mother plants with 2% Benlate improved sterilization procedure. Only one replicate was infected by fungus while the rest were uncontaminated either by fungus or bacteria.

DISCUSSIONS

The standard sterilization method using 70% ethanol and 20% Clorox® was proved insufficient for *in vitro* propagation of field-derived *F. deltoidea*. 100% explants were contaminated by fungus just after 3 days incubation. This observation is foreseeable as it is reported elsewhere that explants taken from the field or *in vivo* materials are difficult and almost impossible to sterilize [12, 13]. Theoretically, explants taken from the field or from plant parts which are located close to the soil are problematic due to the presence of both endophytic and epiphytic

microorganisms. Also, the sources of contaminated cultures are difficult to determine [14] as they may originate from the explants itself or from cross contamination including the laboratory environment such air, people, tools, aseptic technique or even old prepared sterilants solution [14, 15]. Several other factors are the plant species itself, age, explant sources and weather condition.

Failure of the standard sterilization method has led to exploring the use of different sterilants combinations and fungicides. In order to achieve zero contamination rate, the use of Captan, Benlate, Thiram, $HgCl_2$ andantibiotics have been attempted in many studies. In shoot cultures of woody plants, combination of antibiotics such ascefotaxime, tetracycline, rifampicin and polymyxin has been successful to control the bacterial growth [16] while 10% Carbendazim was incorporated as a part of sterilisation procedure in *in vitro* regeneration of *Acmella calva* L [9]. However, in the present study, antibiotics became the last choice because most effective antibiotics against bacteria are toxic to plant material [17] and such routine can be expensive especially at the commercial level.

The main problem in culturing *F. deltoidea* is fungal infection as 80% of infections came from fungus while only 20% were caused from bacteria. The fungal infection was visible as early as three days after incubation while bacterial infection usually took approximately 10 days to show the symptom. In other cases, bacterial infection could be only be seen after four weeks culture but some appeared shortly after subculture. In oil palm tissue culture, leaf explants that were infected by *Fusarium oxysporium* sp *elaeidis* were are passed as healthy due to lack of symptoms as surface sterilization is unable to destroy the fungus but consistently contaminate the culture after many subcultures and transfer [13]. Similar findings have been reported in internal prevalent contaminants that are not detected by visual examination or destroyed by surface sterilization [16].

Both the concentrations and exposure durations to sterilants are crucial in any explant sterilization procedure. If the concentration and exposure duration are too high and long, it will lead to damage and death of plant tissues. If they are too low, it will ineffectively sterilize the explants. The present study has successfully determined the sterilants concentration and exposure duration for *F.deltoidea* sterilization procedure. The results were acceptably good as 78.88% of explants survived. As for pre-treatment of mother plant 100% survival was recorded.

CONCLUSION

It was found that the *in vitro* survival percentage of field-derived explants could be increased from 0% to 78.88%. The optimized sterilization method for field-derived explants of *F.deltoidea* involved the following steps: washing explant with few drops of Teepol and placing it under running tap water for 30 minutes. Next, rinsing one time with dH_2O and soaking in 2% Benlate for about 30 minutes. In a laminar air flow cabinet, the explants should be soaked in 70% EtOH for one to two minutes followed by 0.1% $HgCl_2$ for 5 minutes. Then the explants should be soaked in 25% Clorox® plus a few drops of Tween 20 for another 30 minutes. Finally, the explants should be rinsed with sterile dH_2O for 5 times. For every sterilant substitution from EtOH to $HgCl_2$ or Clorox®, explants should be rinsed for at least two times with sterile dH_2O to eliminate the trace of sterilants that might stick on the explants. To get a better survival percentage of explants up to 100%, it is suggested that the field plants to be pre-treated by spraying 2% Benlate once a week for at least one month before the explants are taken to the laboratory. After the pre-treatment, the field plant should be placed in the greenhouse or at the control environment to maintain the sanitary conditions of the mother plants.

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