Vietnam Journal of Biotechnology **20**(3): 663-673, 2022

EFFECT OF CALCIUM HYPOCHLORITE ON SURFACE STERILIZATION AND SEEDLING GROWTH OF VIETNAMESE COCONUT VARIETIES

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Received: 29.6.2021
Accepted: 04.8.2021

SUMMARY

Coconut (*Cocos nucifera* L.) or 'the tree of life' is one of the most important palm crops due to its versatility. Reduction in coconut productivity due to natural calamity and disease threats has raised the urgent need to develop *in vitro* techniques that can overcome the obstacles of the traditional breeding method. Embryo culture is one of the earliest *in vitro* culture techniques applied to practical problems and so far has proved itself to be of great value to breeders. However, contamination is one of the most serious problems that reduces the efficiency in *in vitro* culture techniques. Thus, appropriate surface sterilization treatments are highly important to *in vitro* culture establishment. The present study was performed to evaluate the sterilization effect of calcium hypochlorite by comparing with sodium hypochlorite. This study also examined the effects of calcium hypochlorite concentrations on plantlets morphogenesis. The findings revealed that amongst the tested treatments, 0.5% (w/v) calcium hypochlorite was the most effective treatment with the lowest rate of contamination. This treatment also significantly improved shoot and root elongation in comparison with calcium hypochlorite at higher concentrations (3% and 6%, w/v). Thus, this concentration was found to be optimal for surface sterilization of two coconut cultivars - Aromatic and Xiem Red Dwarf. Besides that, the results obtained from this study indicated that 70% (v/v) ethanol was not critical in surface sterilization protocol of coconut embryo culture. This research has provided an improved approach for surface sterilization which was previously dependent on sodium hypochlorite and ethanol.

Keywords: calcium hypochlorite, coconut, embryo culture, ethanol, sodium hypochlorite, surface sterilization.

INTRODUCTION

Coconut (*Cocos nucifera* L.) is one of the most essential palm crops, mainly cultivated in the lowlands of different tropical and subtropical countries (Nwite *et al.*, 2017). This species belongs to the palm tree family (*Arecaceae*) and plays an important role as the 'tree of life' since all parts of the tree can be utilized in the production of many important products (Nguyen *et al.*, 2016). For example, it is widely used for food, drink, structural materials and energy supply. In addition, coconut production has contributed tremendously to the national

economy by creating job opportunities for rural workers and increasing income for the farmers (Nguyen *et al*., 2015). Coconut crop has been assessed with the ability to prevent soil erosion, landslide and salinization, which contributes to the development of sustainable agriculture in the future, especially for the low coastal plains.

For the development of new coconut varieties, germplasms have to be collected from the various tropical regions globally, shared through international exchange programs, and then used in breeding programs (Samosir, Adkins, 2014). As the coconut seeds are quite bulky and recalcitrant, *in vitro* culture of zygotic embryos provides a useful alternative for collecting and exchanging germplasms. However, the application of this technique to all cultivars of coconut needs to be optimized.

The *in vitro* conservation of plants comprises a selection of explants, aseptic culture establishment; *in vitro* shooting and rooting followed by acclimatization of the plantlets. Among these stages, the challenging step is a standardization of the sterilization process of explants for aseptic culture establishment. Microbial contamination is one of the most serious problems in plant tissue culture. These microbes compete adversely with the plant tissue cultures for nutrients, and their presence often results in variable growth, tissue necrosis, reduced shoot proliferation, reduced rooting or even culture mortality (Oyebanji *et al*., 2009). Microbes can be readily present in/on the explants or can be reintroduced from poor aseptic handling, unhygienic conditions in the laboratory or from laboratory instruments. Therefore, the surface sterilization process is required to eliminate such contaminants but definitely without affecting the biological activities or normal growth of the explants (Felek *et al*., 2015). The efficiency of disinfection can be influenced by numerous factors such as the physiological state (size, age, and type) of the stock explant, the conditions of working area, and the type of disinfectant and its concentration (Teixeira *et al*., 2016). In case of age, embryos of over-ripe nuts may grow prematurely and disinfection becomes difficult at this stage. Chemicals utilized in disinfection are different or vary in concentrations, doses, applied duration, combination and order of use. Generally, the longer treatment with more concentrated disinfectant(s) is used, the better asepsis results will be achieved. Commonly used disinfectants such as ethanol, mercuric chloride ($HgCl_2$), sodium hypochlorite (NaClO) and calcium hypochlorite ($Ca(ClO)_2$) can help prevent the growth of fungi and bacteria on the culturing media. Ethanol is used to kill microorganisms by either dissolving their membrane lipid bilayer and denaturing their proteins or disrupting the osmotic balance across cell membrane leading to cell death. It effectively eliminates most bacteria, fungi and viruses, except bacterial spores. In different reports, the concentrations of ethanol used are varied. In particular, 95% ethanol is applied for 11 to 12-month-old 'Tagnanan Tall' (Sukendah, Cedo, 2005) and 10 to 12-month-old East African Tall embryos (Muhammed *et al*., 2013). However, high concentrated ethanol or absolute ethanol may just inactivate the microbes without destroying them because the alcohol is unable to fully permeate the microbial membrane. It is believed that a solution of 70% ethanol is more effective than pure ethanol because ethanol relies on water molecules for optimal antimicrobial activity. Therefore, 70% ethanol is applied in most studies and considered as powerful sterilizing agent. Nevertheless, it is also extremely phytotoxic. Because of this, short exposure of ethanol ranging from few minutes to seconds is generally given to the explants (Tiwari *et al*., 2012). Surface sterilization with heavy metal salts is another way for sterilization in which $HgCl_2$ is a major choice. However, due to its toxicity and unsafe nature for both researchers and the environment, this chemical is usually replaced by other disinfectants. NaClO and $Ca(ClO)_2$ are commonly used as sterilizing agents for coconut zygotic embryos with concentrations ranging from 0.5 to 1%. When diluted in water, the hypochlorite salts (NaClO, $Ca(ClO)_2$) lead to the formation of hypochlorous acid (HClO) whose concentration is correlated with bactericidal activity (Nakagawara *et al*.,

1998). $Ca(ClO)_2$ is used for industrial sterilization, bleaching and water purification treatment process. It is relatively stable and has greater available chlorine (up to 65%) than NaClO. Furthermore, even though both chemicals have similar available chlorine contents, $Ca(ClO)_2$ has an initial slower rate of tissue dissolution than NaClO, thus $Ca(ClO)_2$ solution appears to be safer (Dutta, Saunders, 2012). In this study, the aim was to investigate and identify the most efficient sterilization technique for coconut zygotic embryos by comparing the effects of NaClO and $Ca(ClO)_2$ on contaminant removal and plant growth as well as investigating the optimal concentration of $Ca(ClO)_2$.

MATERIALS AND METHODS

Plant materials

The plant materials consisted of zygotic embryos extracted from two varieties, Aromatic (AR) and Xiem Red Dwarf (XRD) coconuts. Coconut fruits (10 to 12 months post pollination) were collected from Ben Tre Province and transported to laboratories at the International University (VNU-HCM) for further processing.

Disinfection and surface sterilization of explants

The fruit was split transversely with a machete to reveal the embryos surrounded by solid endosperm. These embryos were excised from the open nuts using a cork borer and placed in sterile distilled water (Fig. 1A, B). The endosperm enclosing the embryo was washed in 70% ethanol (v/v) for 30 s then rinsed three times with sterile distilled water (Sisunandar *et al.*, 2010). Under aseptic condition, the embryos were isolated from the endosperm and disinfected in five treatments: (1) 0.5% (w/v) NaClO combined with 70% ethanol, (2) 0.5% (w/v) $Ca(ClO)_2$ combined with 70% ethanol, (3) 0.5% (w/v) $Ca(ClO)_2$, (4) 3% (w/v) $Ca(ClO)_2$, and (5) 6% (w/v) $Ca(ClO)_2$. For treatments (1) and (2), the excised embryos were firstly sterilized by ethanol 70% in 1 min and then by the other disinfectant (NaClO in (1) and $Ca(ClO)_2$ in (2)) for 10 min. For treatments (3), (4) and (5), the embryos were disinfected by $Ca(ClO)_2$ with desired concentration for 10 min. After disinfection, all the samples were rinsed with sterile distilled water three times.

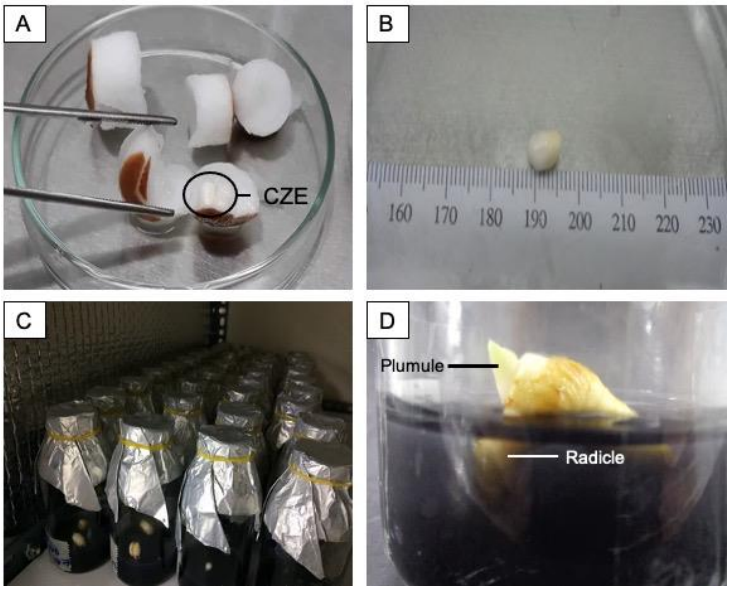

Figure 1. Preparation of plant material. (A) Coconut endosperm plug, (B) Excised embryo from the plug, (C) Cultured embryos in dark chamber, (D) Germinated embryo after 4 weeks in dark chamber. CZE: coconut zygotic embryo.

Culturing media and culturing conditions

Each surface-sterilized embryo was cultured in a 100-mL culture vessel containing 20 mL of Y3 germination medium (GM) (Eeuwens, 1976) with the addition of 45 g L^{-1} sucrose, 6-benzyl aminopurine (BAP) (5 µM), activated charcoal (1 g L^{-1}) (Sigma-USA, C1957) and phytagel (2 g L^{-1}) (Sigma-USA, P8169). The pH was adjusted to 5.8 ± 0.1 with 1 M NaOH solution before autoclaving for 20 min at 121 °C and 1 atm.

For post-germinative development, the plantlets were cultured on Y3 shoot root medium (SRM) including BAP (5 µM), indole 3-butyric acid (IBA) (10 µM), sucrose (45 g L^{-1}), activated charcoal (0.5 g L^{-1}) and phytagel (1.25 g L^{-1}). The pH of the medium was also adjusted to 5.8 ± 0.1 before autoclaving for 20 min at 121 °C and 1 atm.

Evaluation of disinfectant effect on germination

To test the effect of NaClO and $Ca(ClO)_2$ on germination of coconut zygotic embryos, the embryos were cultured in 20-mL of Y3 GM. The cultures were incubated in the dark at 25 °C for 4 weeks (Fig. 1C). Each treatment was replicated three times and the unit for each replicate was 10 embryos. The well-germinated embryos which had plumule visibly and sight of radicle were counted in the germination assay, with data were displayed in the form of a percentage (Fig. 1D). For checking the contamination, bio-hazardous factors such as fungi or bacteria appearing on the surface of the medium or surrounding the embryo were recorded. The percent of contaminated embryos and germinated embryos were determined 4 weeks after initiation.

Evaluation of disinfectant effect on plantlet growth and development

Seedlings from germinated embryos after 4 weeks were sub-cultured in sterile 500-mL growth culture vessels containing SRM and were incubated under illuminating condition. The shoot and root lengths were first measured 6 weeks after initiation and the data were collected every 2 weeks. Each plantlet in the post-germination experiment was considered as one replicate.

Statistical analysis

All data sets were statistically analyzed for variance using *t*-test and ANOVA, Tukey Post Hoc test with P = 0.05 for the significant difference.

RESULTS

Effects of NaClO and $Ca(ClO)_2$ on surface sterilization of coconut zygotic embryos

Regarding contamination rate (Fig. 2A), AR cultivar exhibited 10% of embryos being contaminated by fungi and bacteria in NaClO, which was higher than the ones disinfected by 0.5% $Ca(ClO)_2$ (*ca.* 3.33%). For the other cultivar, the percentage of contaminated XRD embryos in the method using NaClO is lower than the one using $Ca(ClO)_2$ (0% and 3%, respectively). However, statistical analysis showed no significant difference in the rates of contamination and germination between the treatments.

After 4 weeks of incubation in darkness, the cultured embryos of two studied cultivars increased in mass with the appearance of the shoot. Among the treatments, embryos disinfected with 0.5% NaClO and 0.5% $Ca(ClO)_2$ recorded the same germination rates (93.33%) in AR cultivar. Meanwhile, in cultivar XRD, 100% embryos were germinated after surface-sterilized with 0.5% NaClO treatment but only 93.33% embryos germinated with the treatment of 0.5% $Ca(ClO)_2$ (Fig. 2B).

Effect of 70% (v/v) ethanol on surface sterilization of coconut zygotic embryos

As shown in Fig. 3A, AR embryos surface-sterilized with $Ca(ClO)_2$ without ethanol exhibited a higher contamination rate compared to the ones surface-sterilized with $Ca(ClO)_2$ in combination with ethanol. Meanwhile, the exclusion of ethanol from the surface sterilization step yielded a lower percentage of contaminated XRD embryos compared to the

inclusion of ethanol treatment. The two cultivars displayed similar germination rates in the treatment of 0.5% $Ca(ClO)_2$ without ethanol, which was 96.67%. This was slightly higher than the ones in the treatment with both 70% (v/v) ethanol and $Ca(ClO)_2$ (93.33%) (Fig. 3B). However, the difference in contamination and germination rates between these treatments was not significant.

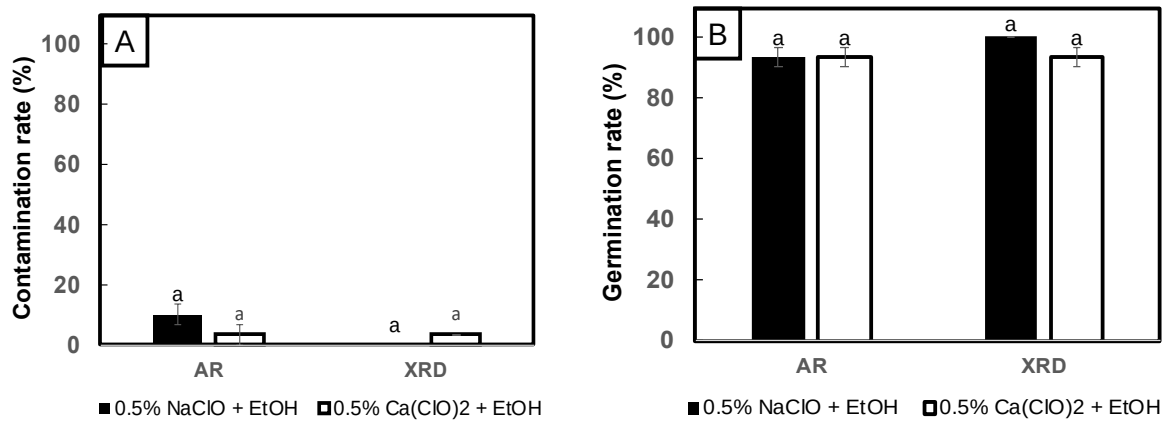

Figure 2. The surface-sterilization effect of 0.5% (w/v) sodium hypochlorite (NaClO) and 0.5% (w/v) calcium hypochlorite ($Ca(ClO)_2$) combined with 70% (v/v) of ethanol (EtOH) based on (A) contamination rate and (B) germination rate in Aromatic (AR) and Xiem Red Dwarf (XRD) coconuts. Different letters in each bar chart depict the significant difference between two treatments of each cultivar. The bars are means of three replications ± SE.

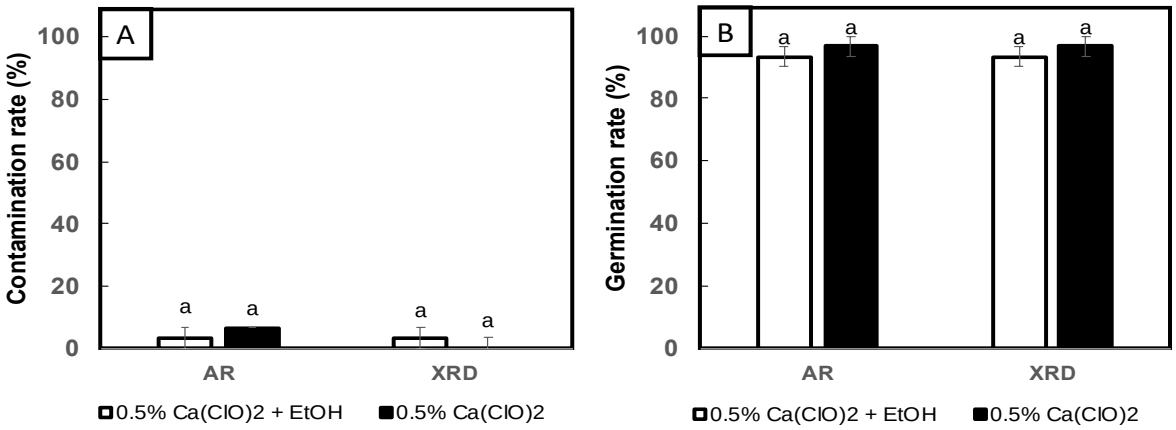

Figure 3. The surface sterilization effect of 70% (v/v) of ethanol (EtOH) based on (A) contamination rate and (B) germination rate in Aromatic (AR) and Xiem Red Dwarf (XRD) coconuts. Different letters in each bar chart depict the significant difference among the treatments. The bars are means of three replications ± SE.

Effect of 70% (v/v) ethanol on plantlet development

As shown in Fig. 4, ethanol application showed negative effects on the growth of both cultivars. In particular, the average shoot length of AR plantlets in treatment with 0.5% $Ca(ClO)_2$ solely was 52.89 mm, which was about two times higher than the ones pretreated with 0.5% $Ca(ClO)_2$ and 70% (v/v) ethanol (28.95 mm) (Fig. 4A). Surface-sterilization with 0.5% $Ca(ClO)_2$ alone also enhanced the average root lengths, which were 37.25 mm for AR and 39.7 mm for

XRD. In contrast, the additional sterilization by 70% (v/v) ethanol lowered the root lengths, which were 6.33 mm for AR and 16 mm for XRD (Fig. 4B).

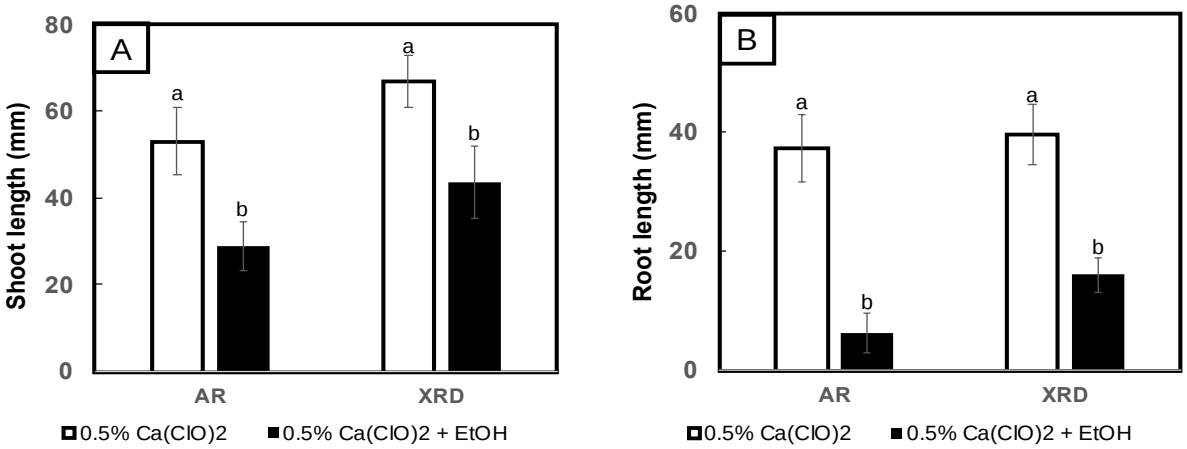

Figure 4. Effect of 70% (v/v) ethanol (EtOH) on plantlet growth after 12 weeks. (A) Shoot length. (B) Root length. Different letters in each bar chart depict the significant difference between the treatment of each cultivar. The bars are means of replications ± SE.

Effect of $Ca(ClO)_2$ at different concentrations on surface sterilization of coconut zygotic embryos

According to our findings, the contamination percentage of AR coconut embryos in surface sterilization by $Ca(ClO)_2$ treatment without 70% (v/v) ethanol was lower than 10% and declined gradually with increasing concentration of $Ca(ClO)_2$ (Fig. 5A). In particular, the contamination rates in treatment of 0.5% $Ca(ClO)_2$, 3% $Ca(ClO)_2$, and 6% $Ca(ClO)_2$ were 6.67%, 3.33%, and 0%, respectively. In contrast, at minimum concentration, 0.5% $Ca(ClO)_2$ was an efficient sterilizing agent with 100% of embryos of XRD found to be contamination-free. While the percentages of contaminated embryos from treatments of 3% and 6% of $Ca(ClO)_2$ increased (to 8.9% and 13.6%, respectively), the difference in contamination rates between these two treatments was not significant (Fig. 5A).

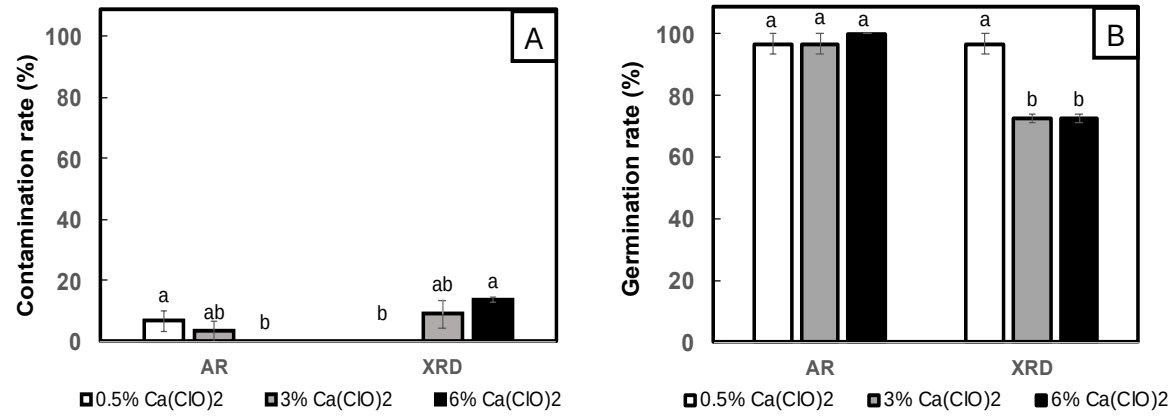

Figure 5. The surface sterilization effect of calcium hypochlorite ($Ca(ClO)_2$) based on (A) contamination rate and (B) germination rate in Aromatic (AR) and Xiem Red Dwarf (XRD) coconuts. Different letters in each bar chart depict the significant difference among the treatments. The bars are means of three replications ± SE.

The obtained results indicated that an increase in $Ca(ClO)_2$ concentration revealed no significant alteration in germination rates of AR embryos. Embryos disinfected by 0.5% $Ca(ClO)_2$ or 3% $Ca(ClO)_2$ showed a germination rate of 96% while for the 6% $Ca(ClO)_2$ application, 100% AR embryos successfully germinated (Fig. 5B). Nevertheless, XRD cultivar showed the negative effects of increased $Ca(ClO)_2$ concentration on the embryo germinability. At the lowest concentration of $Ca(ClO)_2$ (0.5%), the embryos acquired the highest germination rate of 96%. Meanwhile, elevating the concentration of $Ca(ClO)_2$ to 3% and 6% decreased germinability to 72.62% (Fig. 5B). Furthermore, it is noted that the embryos in both cultivars with 0.5% $Ca(ClO)_2$ disinfection had smaller plumule but longer radicle than the corresponding counterparts under 3% and 6% of $Ca(ClO)_2$ treatments.

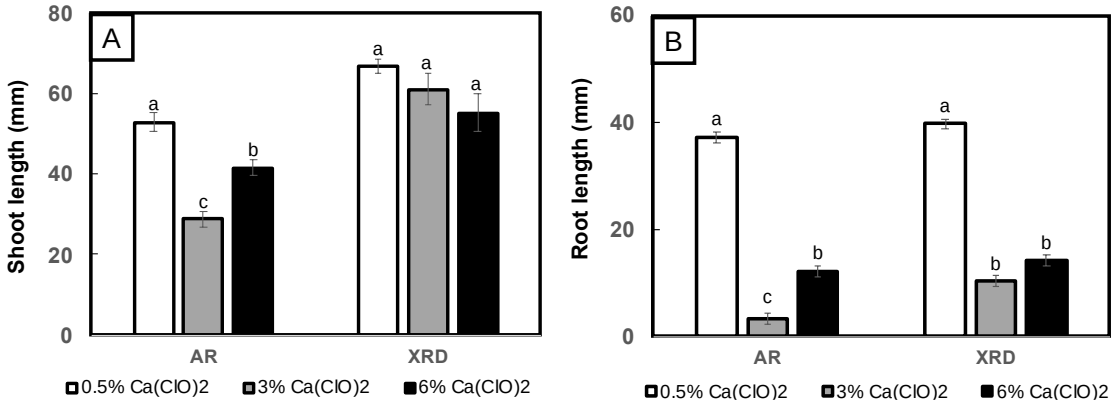

Figure 6. Effect of different concentrations of $Ca(ClO)_2$ in absence of ethanol on plantlet growth after 12 weeks. (A) Average shoot length. (B) Average root length. Different letters in each bar chart depict the significant difference among the treatments. The bars are means of replications ± SE.

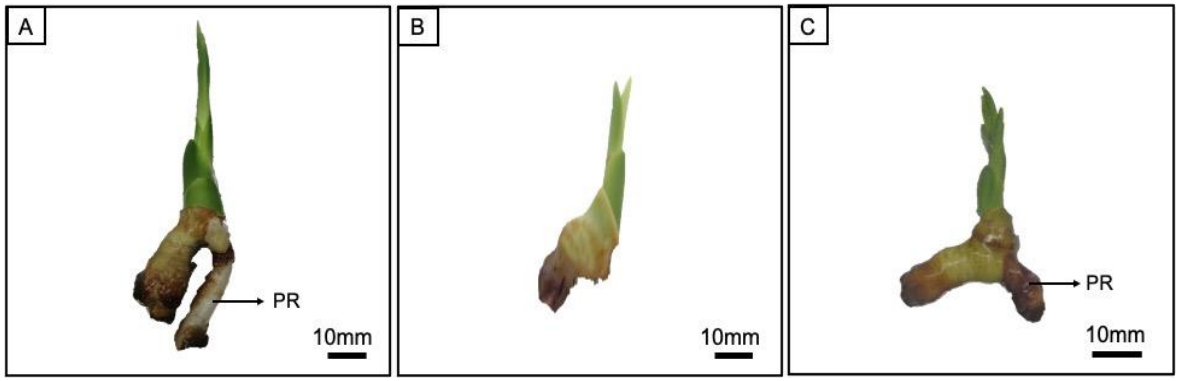

Figure 7. Effect of different concentrations of $Ca(ClO)_2$ in absence of ethanol on Aromatic (AR) plantlet growth and development after 12 weeks. (A) 0.5% $Ca(ClO)_2$. (B) 3% $Ca(ClO)_2$. (C) 6% $Ca(ClO)_2$. PR: primary root.

Effect of $Ca(ClO)_2$ at different concentrations on plantlet development

The results showed that the response to surface sterilization treatments was not found to be cultivar-dependent. In both studied cultivars, the coconut embryos that were surface-sterilized by 0.5% $Ca(ClO)_2$ yielded the highest plantlet growth and development compared with the ones treated with 3% and 6% $Ca(ClO)_2$ (Fig. 6, 7). In particular, the concentration of 0.5% $Ca(ClO)_2$ yielded the highest average shoot lengths which

were 52.89 mm in AR cultivar and 66.8 mm in XRD cultivar (Fig. 6A). AR plantlets in 0.5% $Ca(ClO)_2$ had an average root length of 37.25 mm, which was three times longer than the average root length in the pretreatment with 6% $Ca(ClO)_2$ (12.21 mm), and eleven times longer than that of 3% $Ca(ClO)_2$ (3.30 mm) (Fig. 6B). In addition, AR plantlets pretreated with 0.5% $Ca(ClO)_2$ developed adventitious roots while plants in other treatments possessed only one primary root (Fig. 7). The XRD plantlets also showed a similar trend to the AR ones. The root length in the method using 0.5% $Ca(ClO)_2$ was the highest and well-developed while the growth of root in the treatments of higher concentrations of $Ca(ClO)_2$ were significantly lower (Fig. 6B).

DISCUSSION

Maintenance of an aseptic condition is a prerequisite for success of *in vitro* embryo germination and proliferation. The zygotic embryo's survival is highly influenced by the concentration of applied disinfectant, the immersion duration and the combination of pre-disinfection and disinfection. All datasets in this study indicate that NaClO and $Ca(ClO)_2$ possess similar effects on the survival ability of coconut embryos, while 70% (v/v) ethanol does not play a key role in surface-sterilization protocol (Fig. 3).

Ethanol is an extremely phytotoxic sterilizing agent. For this reason, the plant material should be exposed to ethanol for a short time. To improve the effectiveness of the sterilization procedure, ethanol is generally used before treatment with other compounds. It has been reported that alcohols are rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria; they also are tuberculocidal, fungicidal, and virucidal but do not destroy bacterial spores (Bloomfield *et al*., 1991). Ethanol cidal activity drops sharply when it is diluted below 50% in concentration. The optimum bactericidal concentrations for ethanol should be 60 to 90% solution in water (Gross, 1987).

Apart from ethanol, it is reported that when diluted in water, the hypochlorite salts used (NaClO, $Ca(ClO)_2$) lead to formation of HClO, whose concentration is correlated to bactericidal activity (Nakagawara *et al*., 1998). The effectiveness of HClO as a disinfecting agent stems from the small size of the molecule and its electrical neutrality, which allow ready penetration into cells. HClO is found to effectively destroy fungi by lysing their cell wall and binding with the unsaturated lipid layer (cell membrane), leading to subsequent disruption of cellular integrity. Likewise, hypochlorite has been known as an effective compound to kill bacteria; even at micromolar concentrations (Nakagawara *et al*., 1998). However, little is known about the exact mechanisms of bacterial killing by this sanitizer. Previous studies suggested that HClO could kill bacteria as it leads to lethal DNA damage (Dukan, Touati, 1996; Wlodkowski, Rosenkranz, 1975). Furthermore, as reported in EU Regulation (EC) No 1272/2008, NaClO is a caustic chemical that can cause injury when it contacts living tissues. Also, ocular exposure to NaClO may cause irritation, pain, lacrimation, photophobia and retinitis. Thus, the application of $Ca(ClO)_2$ can ensure better safety for the technicians. In addition, $Ca(ClO)_2$ is easy to store and handle with its appearance in powder form, whereas NaClO is in liquid form. To conclude, with similar surface sterilization efficiency, $Ca(ClO)_2$ can be a more favorable reagent for surface sterilization of coconut embryos.

Calcium ion is a macronutrient that is a crucial regulator of growth and development in plants. It should be noticed that even embryos are washed by sterile distilled water after dipping in $Ca(ClO)_2$, it could not remove all $Ca(ClO)_2$ residues from these samples. Fortunately, Ca^{2+} plays a crucial role in determining the structural rigidity of the plant cell wall (Burstrom, 1968; Jones, Lunt, 1967). It has also been known for many years that Ca^{2+} plays an important role in controlling membrane structure and function (Burstrom, 1968; Jones, Lunt, 1967). A general idea is that Ca^{2+} ions, by binding to

phospholipids, stabilize lipid bilayers and thus provide structural integrity to the cellular membrane. From a physiological point of view, a frequent observation has been that Ca^{2+} controls membrane permeability (Epstein, 1972; Bonomelli *et al*., 2018). It had been shown that a low $[Ca^{2+}]_e$ caused a decline in the ability of soybean and maize root tissue to absorb and retain solutes (Hanson, 1960). Thus, an appropriate amount of Ca^{2+} can enhance the ability of root function. A preliminary study on disinfection of *S. biafrae* nodal explants proposed that treatment of 70% ethanol for 20 s and 10% $Ca(ClO)_2$ for 15 min was the best surface-sterilization protocol as it gave 90% growth of the plant compared with 60% growth recorded in the treatment of 70% ethanol for 3 min and 10% $Ca(ClO)_2$ for 20 min (Bello *et al*., 2018). These properties might support the plants to grow steadily. Thus, $Ca(ClO)_2$ is not as simple as a disinfectant, it could support plant development as well.

Chlorine is an essential micronutrient for higher plants, but this element could bring some negative effects to plant growth. Experiments with the crop *S. alba* confirmed that chlorine ions are generally toxic to plant growth, even at relatively low concentrations, and may cause irreversible damage to plant development. Chlorine enters the plants through the roots by a symplastic pathway and is mobile within the plant. The intake process influences fluxes and accumulation of the chlorine in the plants and its distribution within the plants. The fluxes across membranes and between tissues limit growth and water translocation in the plants as well as interfere with photosynthesis through inhibition of photosynthetic pigment production (Fargašová, 2017). In addition, it has been shown that a chlorine cover has been formed during hypochlorite disinfection of rice seeds and this artifact could not be eliminated simply by rinsing the seeds, even thoroughly, with sterile distilled water. This phenomenon might impede the disinfectant ability (Miché, Balandreau, 2001). Furthermore, it seems that the bactericidal activity of chlorine is due to formation of secondary products, as hypochlorous acid reacts avidly with a wide variety of subcellular compounds (membranes, proteins, etc.) (Dukan, Touati, 1996). In particular, HClO reacts with NH_4^+ (which is one component in plant growth medium) and organic amines to form highly toxic chloramines, which could be the actual killing agents. These chloramines are very diffusible species that can enter cells through membranes and react with intracellular components, including DNA (Shih, Lederberg, 1976; Thomas *et al*., 1987).

For these lines of evidence, it is reasonable to explain that plantlet development is inhibited as $Ca(ClO)_2$ concentration increases. Nevertheless, Sisunandar *et al*. (2018) reported that the coconut seedlings in the 6% $Ca(ClO)_2$ treatment were well-developed. This situation might occur due to a larger amount of Ca^{2+} in 6% than 3% treatment supporting plant growth and overcoming the negative effect from HClO. Meanwhile, Ca^{2+} in treatment 3% was not enough to hold up the plants against those side-effects of chlorine released. However, plantlets in 0.5% $Ca(ClO)_2$ were significantly well-developed comparing with those in 6% $Ca(ClO)_2$ while the contamination issue can be under control. Thus, 0.5% $Ca(ClO)_2$ seems to be the optimal concentration for AR and XRD coconut plantlet's development.

CONCLUSION

Calcium hypochlorite placed a significant effect on the growth and development of coconut zygotic embryos. The obtained results also showed that ethanol was not a critical disinfectant in surface sterilization protocol. In this study, the optimal protocol for coconut embryo surface sterilization was 0.5 % (w/v) calcium hypochlorite, which yielded higher percentage of survival ability, length of shoot, length of root and number of roots. Another finding is that the pretreatment with 0.5% $Ca(ClO)_2$ is not cultivar-dependent. Therefore, it can be widely applied to various coconut cultivars, which provides large support in

successful *in vitro* coconut culture.

Acknowledgement: *The research project is funded by Vietnam's Ministry of Science and Technology through research grant number ĐTĐL.CN-12/19.*

REFERENCES

Bello OA, Esan, Edward B, Obembe, Olawole O (2018) Establishing surface sterilization protocol for nodal culture of *Solanecio biafrae*. *IOP Conf Ser: Earth Environ Sci* 210: 012007.

Bloomfield SF, Arthur M, Looney E, Begun K, Patel H (1991) Comparative testing of disinfectant and antiseptic products using proposed European suspension testing methods. *Lett Appl Microbiol* 13(5): 233–237.

Bonomelli C, Arias MI, Villalobos L (2018) Adaptation and validation of a methodology for the measurement of calcium fractions in fruits. *Comm Soil Sci Plant Anal* 49(6): 735–744.

Burstrom HG (1968) Calcium and plant growth. *Biol* 43(3): 287–316.

Dukan S, Touati D (1996) Hypochlorous acid stress in *Escherichia coli*: Resistance, DNA damage, and comparison with hydrogen peroxide stress. *J Bacteriol Res* 178(21): 6145–6150.

Dutta A, Saunders WP (2012) Comparative evaluation of calcium hypochlorite and sodium hypochlorite on soft-tissue dissolution. *J Endod* 38(10): 1395–1398.

Epstein E (1972) Mineral nutrition of plants: Principles and perspective. Wiley Publisher, NY.

Eeuwens CJ (1976) Mineral requirements for growth and callus initiation of tissue explants excised from mature coconut palms (*Cocos nucifera*) and cultured *in vitro*. *Physiol Plant* 36(1): 23–28.

Fargašová A (2017). Plant stress activated by chlorine from disinfectants prepared on the base of sodium hypochlorite. *Nova Biotechnol et Chim* 16: 76–85.

Felek W, Firew M, Belayneh A (2015) Optimization of explants surface sterilization condition for field grown peach (*Prunus persica* L. Batsch cv. Garnem) intended for *in vitro* culture. *Afr J Biotechnol* 14: 657–660.

Hanson JB (1960) Impairment of respiration, ion accumulation, and ion retention in root tissue treated with ribonuclease and ethylenediamine tetraacetic acid. *Plant Physiol* 35(3): 372–379.

Jones RGW, Lunt OR (1967) The function of calcium in plants. *Bot Rev* 33(4): 407–426.

Magdalita PM, Damasco OP, Adkins SW (2010) Effects of medium replenishment and acclimatization techniques on growth and survival of embryo cultured coconut seedlings. *Philipp Sci Lett* 3: 1–9.

Miché L, Balandreau J (2001) Effects of rice seed surface sterilization with hypochlorite on inoculated *Burkholderia vietnamiensis*. *Appl Environ Microbiol* 67(7): 3046–3052.

Muhammed N, Nyamota R, Hashim S, Malinga JN (2013) Zygotic embryo *in vitro* culture of *Cocos nucifera* L. (sv. East African Tall variety) in the coastal lowlands of Kenya. *Afr J Biotechnol* 12(22): 3435–3440.

Nakagawara S, Goto T, Nara M, Ozawa Y, Hotta K, Arata Y (1998) Spectroscopic characterization and the ph dependence of bactericidal activity of the aqueous chlorine solution. *Anal Sci* 14(4): 691–698.

Nguyen KB, Le TT, Bui TV (2015) Fruit growth and lipid accumulation in the solid endosperm of Ta Xanh coconut cultivar (*Cocos nucifera* L.). *Sci Technol Develop* 18: 64–74.

Nguyen QT, Bandupriya, HDD, Foale M, Adkins SW (2016) Biology, propagation and utilization of elite coconut varieties (makapuno and aromatics). *Plant Physiol Biochem* 109: 579–589.

Nwite PA, Ikhajiagbe B, Owoicho I (2017) Germination response of coconut (*Cocos nucifera* L.) zygotic embryo. *JASEM* 21(6): 1019–1021.

Oyebanji O, Nweke O, Odebunmi O, Galadima, N, Idris M, Nnodi U, Afolabi A, Ogbadu G (2009) Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *Afr J Biotechnol* 8(20): 5395–5399.

Samosir YMS, Adkins S (2014) Improving acclimatization through the photoautotrophic culture of coconut (*Cocos nucifera*) seedlings: An *in vitro* system for the efficient exchange of germplasm. *In Vitro Cell Dev Biol Plant* 50(4): 493–501.

Sisunandar, Rival A, Turquay P, Samosir Y, Adkins SW (2010) Cryopreservation of coconut (*Cocos nucifera* L.) zygotic embryos does not induce

morphological, cytological or molecular changes in recovered seedlings. *Planta* 232(2): 435–447.

Sisunandar, Alkhikmah, Husin A, Julianto T, Yuniaty A, Rival A, Adkin SW (2018) *Ex vitro* rooting using a mini growth chamber increases root induction and accelerates acclimatization of Kopyor coconut (*Cocos nucifera* L.) embryo culture-derived seedlings. *In Vitro Cell Dev Biol Plant* 54(5): 508–517.

Sukendah S, Cedo ML (2005) *In vitro* conservation of coconut (*Cocos nucifera* L.) embryos in culture media. *BIOTROPIA* 25: 11–21.

Teixeira da Silva JA, Winarto B, Dobránszki J, Cardoso JC, Zeng S (2016) Tissue disinfection for preparation of *Dendrobium in vitro* culture. *Folia Hortic* 28(1): 57–75.

Tiwari S, Arya A, Kumar S (2012) Standardizing sterilization protocol and establishment of callus culture of sugarcane for enhanced plant regeneration *in vitro*. *Res J Bot* 7(1): 1–7.

Wlodkowski TJ, Rosenkranz HS (1975) Mutagenicity of sodium hypochlorite for *Salmonella typhimurium*. *Mutat Res* 31(1): 39–42.