RESEARCH ARTICLE
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In vitro sterilization and shoot induction of durian cv. Chani (*Durio zibethinus* Murr.) using chlorine dioxide and plant growth regulators

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ABSTRACT: Tissue culture of durian remains a bottleneck problem due to difficulty in surface sterilization and regeneration of initial explants caused by mucilage secretion after explant wounding. Chlorine dioxide (ClO_2) is a disinfectant and can be used for plant tissue culture instead of the autoclaving medium. The aim of this research was to study effects of ClO_2 and plant growth regulators (PGRs) on *in vitro* sterilization and shoot induction. *Ex vitro* nodal segment was cut and directly transferred to the medium with different concentrations of ClO_2 compared to the autoclaving medium. The results showed that 90 mg/l ClO_2 gave the best results in sterilization at 45.45% and axillary bud break at 60%. For concentrations of N^6-benzyladenine (BA) tested, the highest axillary bud break at 71.42% was obtained from 1 mg/l BA-containing medium after 4 weeks of culture. When transferring sterile axillary shoot to gibberellic acid (GA_3)-containing culture medium of different types, the results demonstrated that shoot elongation was successful from culturing on solid medium overlaid with liquid medium with the same components together with 1 mg/l GA_3 for 4 weeks. It can be concluded that ClO_2 improves sterilization of culture medium and explants. BA together with GA_3 promote shoot development of durian cv. Chani.

KEYWORDS: durian, ClO_2 , sterilization, shoot induction

INTRODUCTION

Durian (*Durio zibethinus* Murr.) is well known as the king of fruit and popularly grown and consumed in many countries including Thailand [1]. It is a high-value, tropical fruit well known for its unique, pungent smell and sweet and creamy flesh [2]. Several cultivars of durian including Mon Thong, Chani, Kan Yao, etc., which are popular for consumption and exportation. Chani is an indigenious variety that is notable and has considerable production potential. However, the cultivated area for this variety is limited due to urbanization and environmental changes leading to insufficient planting areas [1]. Traditionally, durian is normally propagated by sexual method through seeds leading to unstable genetic fidelity because it is a cross pollinated crop. Currently, asexual propagation by grafting is widely used, however, this method limits the number of plants that can be produced. Tissue culture can be used to solve the problem and it is successful in many perennial plants including fruit trees in the family Malvaceae. However, the success of *in vitro* propagation of durian has not been reported. Thus, it is an urgent need to work on this issue. Normally, for tissue culture of perennial plants that do not affect by gum, phenol is produced from wounding thus making easy to micropropagate in both organogenesis and embryogenesis. On the other hand, some plants such as rubber tree, mangosteen, jackfruit etc. produce different chemical compounds when the explants are sectioned. Nevertheless, shoot induction and proliferation of callus and adventitious shoots or multiple shoots could be obtained. In case of durian, there are few reports on direct shoot induction by axillary bud. 6-Benzylaminopurine (BAP) was identified as the optimal plant growth regulator (PGRs) for promoting shoot organogenesis, exhibiting superior efficacy in stimulating the initiation, subsequent proliferation, and biomass accumulation of shoots [3]. Namhomchan [4] reported that woody plant medium (WPM) with 1 mg/l BA can induce shoot but it cannot develop into complete plantlet resulting in lacks of information of suitable PGRs on shoot formation of durian. Our previous study (unpublished data) reported that the main problem of tissue culture of durian is mucilage secretion after tissue wounding which significantly increases when sterilized, leading to high contamination and difficulty in plantlet regeneration. Disinfestation by adding chemical directly into culture medium is an alternative method that is postulated to reduce mucilage production. There are several disinfectants such as sodium hypochlorite, calcium hypochlorite, chlorinated trisodium phosphate, and chlorine dioxide [5]. From preliminary study, ClO_2 is effective in surface sterilization of durian (unpublished data). The concentrations depend on plant species and explant types.

ClO_2 is classified as an A1-level disinfectant. It is a potent oxidizer that disrupts bacterial cell walls and easily diffuses across the surface of microbial membrane [5]. It has been widely used for surface sterilization because of its disinfection efficiency and safety. Its application has been flexible to plant tissue culture [6]. ClO_2 was used as a disinfectant for surface sterilization of explants by immersing the explants in 160 mg/l ClO_2 for 15 to 40 min that promotes adventitious shoot in *Crassula portulacea* at 30 shoots/explant within 21 days of culture [7]. In chrysanthemum, 10 µg/l ClO_2 achieved one-step regeneration by inducing the growth of roots and shoots within 3 weeks [6]. Therefore, the objective of this research was to investigate the effects of ClO_2 and PGR on *in vitro* direct shoot regeneration of durian cv. Chani.

MATERIALS AND METHODS

Plant materials, culture medium and condition

One-year old of healthy plants of durian cv. Chani was selected and used as source of explants for establishing the aseptic materials. The plant was grown and maintained at Crop Biotechnology Laboratory, Agricultural Innovation and Management Division, Faculty of Natural Resources, Prince of Songkla University, Songkhla, Thailand. The plants were watered twice a day and weekly applied the 15-15-15 fertilizer at 10 g/plant.

Shoot induction medium (SIM); 1/2MS together with 1/2WPM supplemented with 1 mg/l BA, 3% sucrose, adjusted to pH 5.7 and solidified with 0.6% agar. The agar medium was melted in microwave and leaved at room temperature to cool down at approximately 50–60 °C. Then ClO_2 was added into the medium to make final concentration as desirable. For ClO_2 preparation, it was prepared as stock solution (4 g/l) by dissolving in distilled water and stored at 4 °C under dark condition until use.

For culture conditions, the cultures were maintained at 26 ± 2 °C under 10 h photoperiod at light intensity of 25 µmol/m^2/s provided with cool-white, fluorescent lamp.

Shoot induction

1. Effect of concentrations of ClO_2 on sterilization and axillary bud induction

The node from *ex-vitro* grown plant was used as starting material. It was cut (approximately 1–2 cm long) and directly cultured without disinfestation on SIM with various concentrations of ClO_2 (45, 90, 135 mg/l) without autoclaving in comparison with control treatment (autoclaved medium and surface steriled explant by soaking in 20% Clorox for 20 min). After 4 weeks of culture, the sterile culture percentage, axillary bud break and characteristics of shoot were investigated.

Sterilization (%) = [(The number of cultured explants without contamination)/(The total number of cultured explants)] × 100.

Axillary bud break (%) = [(The number of explants that produced axillary bud)/(The total sterile nodal explants)] × 100.

2. Effect of concentrations of BA on sterilization and shoot induction

The node (approximately 1–2 cm long) from ex-vitro grown plant was cut and directly cultured without disinfestation on SIM without autoclaving for 4 weeks. SIM was supplemented with various concentrations of BA and 90 mg/l ClO_2. The positive control was 1 mg/l BA and 0.5 mg/l NAA, as obtained from preliminary study. The results in term of percentage of axillary bud break and shoot characters were recorded.

3. Effect of GA_3 containing different types of culture medium on shoot elongation

The sterile node was transferred to SIM supplemented with 1 mg/l BA together with GA_3-containing culture medium of different types: solidified SIM without GA_3, solidified SIM with 1 mg/l GA_3, and solidified SIM overlaid with 1 mg/l BA and 1 mg/l GA_3-containing liquid medium. After 4 weeks of culture, the shoot formation, shoot length and shoot characters were recorded.

Experimental design and statistical analysis

Completely randomized design (CRD) was performed with 5 replications. Each replication consisted of 3 tubes. The means among treatments were compared using Duncans multiple range test (DMRT) in experiment 1 and least significant difference (LSD) in experiments 2, 3 at 1% or 5% probability. The experimental data were analyzed using R 2.14.0 software and subjected to one-way analysis of variance (ANOVA).

RESULTS

Effect of different concentrations of ClO_2 on sterile percentage and axillary bud break

The results showed that high concentration of ClO_2 resulted in high sterile percentage. ClO_2 at 135 mg/l which is the highest concentration tested gave the highest sterile percentage at 50%, which was not significantly different from 90 mg/l ClO_2 but was significantly different from the positive control and 45 mg/l ClO_2. Increase in concentrations of ClO_2 up to 90 mg/l resulted in high percentage of axillary bud break and concentrations at higher than this gave low percentage of axillary bud break. There are significantly different responses in axillary bud induction among all concentrations of ClO_2 tested and the highest result at 60% was obtained from 90 mg/l ClO_2-containing medium (Table 1). However, all treatments provided only one axillary bud/explant.

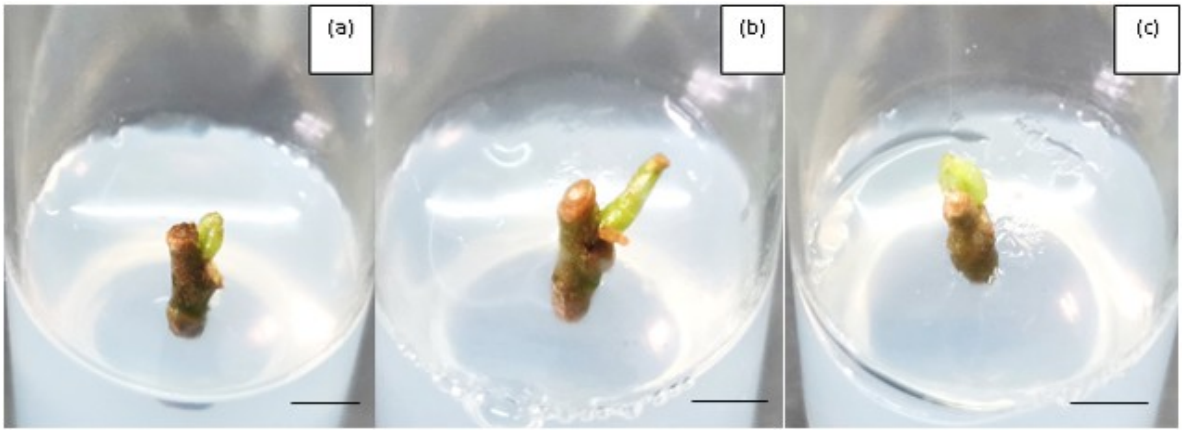

Fig. 1 Characteristics of axillary bud of durian cv. Chani obtained from SIM medium supplemented with different concentrations of BA together with 90 mg/l ClO_2 for 4 weeks (bar = 0.5 cm): (a) 1 mg/l BA + 0.5 mg/l NAA (positive control); (b) 1 mg/l BA; (c) 2 mg/l BA.

Table 1 Effect of concentrations of ClO_2-containing SIM on sterilization and axillary bud induction of durian cv. Chani for 4 weeks.

Conc. of ClO_2 (mg/l)	Sterilization (%)	Axillary bud break (%)	No. of axillary bud (bud/explant)
Positive control (20% Clorox; 20 min)	16.67 ± 1.05^{b}	20.00 ± 1.58^{c}	1.00
45	0.00 ± 0.00^{c}	0.00 ± 0.00^{d}	1.00
90	45.45 ± 1.84^{a}	60.00 ± 4.18^{a}	1.00
135	50.00 ± 3.65^{a}	33.33 ± 3.30^{b}	1.00
F-test	**	**	
C.V. (%)	18.02	20.78	

**Significantly different ($p < 0.01$); Percent coefficient of variation (C.V. (%)); Mean values followed by the same letter within a column are not significantly different, according to DMRT.

Table 2 Effect of concentrations of BA-containing SIM with 90 mg/l ClO_2 on axillary bud break of durian cv. Chani for 4 weeks.

Conc. of BA (mg/l)	Axillary bud break (%)	No. of axillary bud (bud/explant)
Positive control (1 mg/l BA+0.5 mg/l NAA)	22.50 ± 3.65^{b}	1.00
1	71.42 ± 3.73^{a}	1.00
2	25.00 ± 2.88^{b}	1.00
F-test	**	
C.V. (%)	23.07	

**significantly different ($p < 0.01$); Percent coefficient of variation (C.V. (%)); Mean values followed by the same letter within a column are not significantly different, according to LSD.

Effect of concentrations of BA on axillary bud break

Different concentrations of BA resulted in different results in axillary bud break. The results demonstrated that 1 mg/l BA gave the highest axillary break at 71.42% significant difference with positive control. For number of axillary buds, the results showed that only one shoot was obtained from all treatments (Table 2). The axillary buds obtained from all treatments were small and green, except those from the medium supplemented with 1 mg/l BA, which showed greater shoot elongation (Fig. 1).

Table 3 Effect of GA_3-containing SIM with 1 mg/l BA on shoot elongation of durian cv. Chani for 4 weeks.

Treatment	Shoot formation (%)	No. of shoot (shoot/explant)	Shoot length (mm)
Control	33.33 ± 0.00^{b}	1.00	5.00 ± 0.57^{b}
Solid medium	66.67 ± 8.33^{a}	1.00	6.67 ± 0.81^{ab}
Overlay with 1 mg/l GA_3	80.00 ± 3.16^{a}	1.00	8.00 ± 0.54^{a}
F-test	**		*
C.V. (%)	13.79		18.45

*, **significantly different ($p < 0.05$, 0.01); ns = not significantly different; Percent coefficient of variation (C.V. (%)). Control = solidified SIM without GA_3; Solid medium = solidified SIM with 1 mg/l GA_3; Overlay with 1 mg/l GA_3 = solidified SIM overlaid with 1 mg/l BA and 1 mg/l GA_3-containing liquid medium; Mean values followed by the same letter within a column are not significantly different, according to LSD.

Effect of GA_3-containing culture medium of different types on shoot elongation

Significantly difference result in shoot elongation was obtained between cultured medium with and without GA_3. The results showed that solid medium overlaid with liquid medium containing 1 mg/l GA_3 gave the highest shoot elongation at 80% followed by solid medium with 1 mg/l GA_3 and solid medium without GA_3 that provided shoot elongation at 66.67 and 33.33%, respectively. For number of shoots, all culture

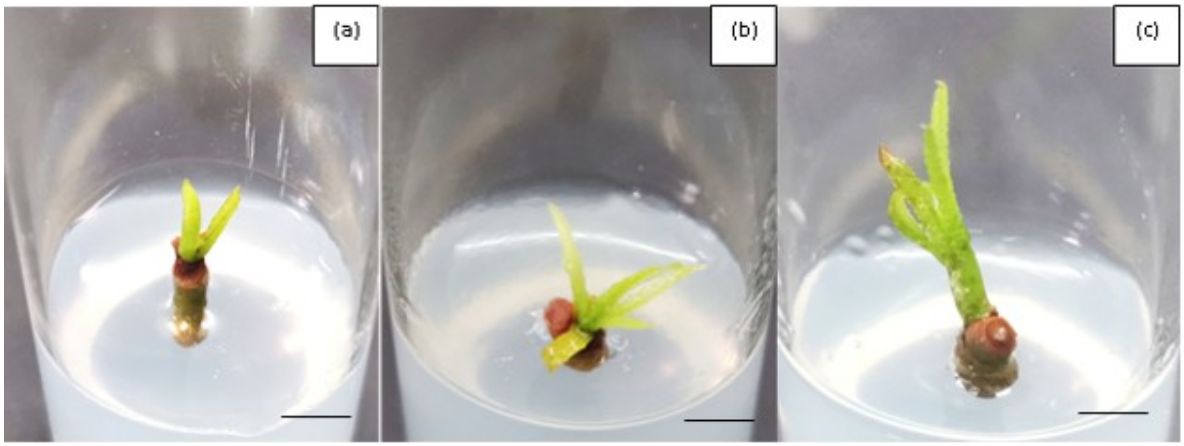

Fig. 2 Characteristics of shoot of durian cv. Chani cultured on SIM medium supplemented with 1 mg/l BA without ClO_2 for 4 weeks (bar = 0.5 cm): (a) without GA_3; (b) solid medium + 1 mg/l GA_3; (c) solid medium overlaid with 1 mg/l BA and 1 mg/l GA_3.

media tested provided only one shoot/explant. However, there are significant differences in shoot length. The highest length of shoot at 8 mm was obtained from solid medium overlaid with liquid medium with 1 mg/l GA_3 followed by solid medium with 1 mg/l GA_3 (6.67 mm) and solid medium without GA_3 (5 mm) (Table 3; Fig. 2).

DISCUSSION

Aseptic technique is one of the most important factors for successful *in vitro* culture. Contamination during plant tissue culture processes results in the loss of operating budgets and plant materials [8]. In perennial plants, there are many problems for *in vitro* propagation such as phenolic compound, gum and mucilage secretion. In durian, we found that the main problems are mucilage secretion following explant wounding which is produced in greater quantities when sterilized it using standard protocol (20% Clorox for 20 min). This mucilage causes high contamination rates in cultured explant which affects the success of *in vitro* regeneration of durian. Conversely, sterilization without soaking explant in liquid solution can solve these problems and improve the responses of initial explants. ClO_2 is an environmentally friendly chemical that has been extensively applied in various aspects: medium sterilization [9], and disinfection of explants [10]. ClO_2 was applied in some plant species such as banana [11], chrysanthemum [6], gerbera [12], potato [9], philodendron [13] etc. However, there are only few reports on *in vitro* regeneration in perennial plants. Moreover, there is no report on the use of ClO_2 to sterilize *ex-vitro* grown explant and culture medium, making this tissue culture approach unique in that it eliminates both autoclaving of the medium and standard explant sterilization. High concentrations of ClO_2 resulted in high sterilization rates. The optimum ClO_2 concentration for durian is 90 mg/l due to high percentage of sterilization and axillary bud responses. Whereas concentrations at higher than 90 mg/l are toxic to plant and also caused severe browning. While the highest number of shoots of Persian violet were obtained on 5 mg/l ClO_2 after culturing for 4 weeks [14]. In case of *Anubias* sp. 'White', ClO_2 at 100 mg/l can reduce complete contamination and increase survival rate at 100% after 8 weeks of culture [15]. The optimal concentration varies depending on plant species. ClO_2 may influence reactive oxygen species (ROS) accumulation and turnover through its strong oxidizing properties, further altering the shoot organogenesis ability of plants [7].

Direct organogenesis has been conducted by adding exogenous PGRs into culture media, especially cytokinins. BA is one type of cytokinins and widely used in shoot induction and proliferation in many plant species such as banana [16], caladium [17], Chinese toon [18], highbush blueberry [19]. For durian, the results showed that the axillary bud initially sprouted from nodal segment within 2 weeks of culture but it has slow growth and difficult to develop into new shoot. Among BA concentrations tested, 1 mg/l gave the best results in axillary bud induction. However, significant differences were not observed in number of shoots after 4 weeks of culture. Contrary results were obtained by Gonbad et al [20] who reported that 3 mg/l BA-containing medium gave the best results in shoot induction of Iran 100 tea clone. The highest shoot regeneration at 93.33% and number of shoots at 1.87 shoots/explant of olive was obtained from 4 mg/l BA-containing medium [21]. In *Magnolia* 'Vulcan', it was found that 0.5 mg/l BA-containing medium was suitable for shoot induction with 5.9 shoots/explant [22]. The suitable concentrations of BA for shoot induction differ depending on the species [22].

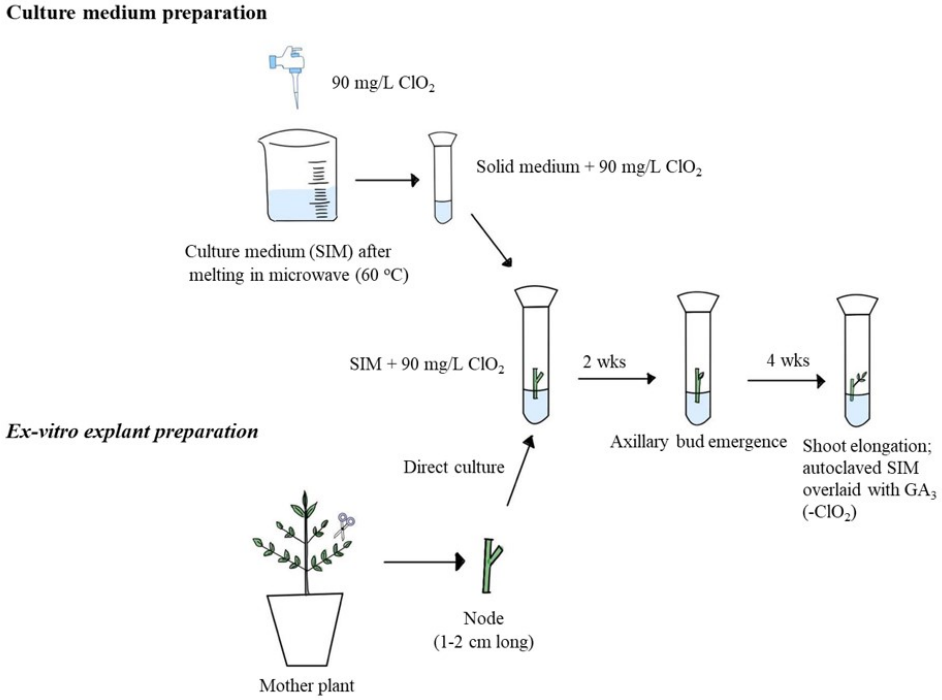

Fig. 3 Summary of *in vitro* sterilization and shoot induction of durian cv. Chani using ClO2.

The most important mechanism of cytokinins is to stimulate shoot multiplication and inhibit their elongation [20]. The shoots obtained from all concentrations of BA were tiny shoot and cannot elongate after 4 weeks of culture. Upon transferring sterile shoots to GA_3-containing culture medium of different types, the results showed that elongated shoot was successful from medium overlaid with liquid medium containing 1 mg/l BA and 1 mg/l GA_3. Application of GA_3 is an effective way to alter plant height by regulating cell elongation and internode elongation [23]. Contrary results were obtained by Geng et al [24] who reported that 0.5 mg/l GA_3 in combination with 2 mg/l BA gave the highest shoot elongation at 93% in apple. However, there is no report on *in vitro* shoot culture overlaid with GA_3. In addition, a greater understanding of the GA_3 effectiveness and interactions between concentrations of GA_3 and cytokinins may improve propagation of plant [24]. From this research, *in vitro* sterilization percentage and shoot induction of durian using ClO_2 were successful. This method is easy and reduces steps of plant micropropagation (Fig. 3) and has been submitted for patent protection in Thailand (Patent Request No. 2301006669). The efficacy of this protocol for explant sterilization and shoot initiation is expected to be transferable to other durian cultivars, especially those of commercial interest, such as 'Monthong'. In the next step, shoot proliferation in durian is being developed not only for mass propagation but also for *in vitro* conservation.

CONCLUSION

The *in vitro* sterilization of nodal explants and shoot induction of durian cv. Chani was successfully carried out by adding 90 mg/l ClO_2 into the culture medium without autoclaving. Shoot elongation was achieved from the autoclaved medium overlaid with 1 mg/l GA_3.

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