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Effective Surface Sterilization Method using Plant Preservatives Mixture and Shoot Multiplication of *Clinacanthus nutans*

Florisa Landa, Yvonne C. Chia, Roslin Ombokou and Zaleha A. Aziz*

Faculty of Science and Natural Resources, University Malaysia Sabah, Jalan UMS, 88400 Kota Kinabalu, Sabah, Malaysia

**Corresponding author*

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A B S T R A C T

Effective surface sterilization and shoot regeneration protocols for *Clinacanthus nutans* are described. Nodal segments of *C. nutans* were sterilised by single- and double-stage sterilisation methods. In the double-stage method, explants were soaked in Plant Preservative Mixture (PPM) at various concentrations (0, 1.25, 2.50, 5.0 µL/mL). For direct shoots regeneration, MS media were supplemented with TDZ, BAP, or KN in single or in combination (BAP+NAA or TDZ+NAA). For indirect shoot regeneration, single application (BAP or KN) and combination treatment (TDZ+BAP or TDZ+KN) of cytokinins were evaluated. The use of PPM (1.25 µL/mL) resulted in survival rate of explants at 82 ± 4.5 %. Direct shoot multiplication was the highest on the medium with 1.0 mg/L BAP + 0.1 mg/L TDZ where explants produced 20 ± 1.5 % primary, 57 ± 6.2 % secondary and 77 ± 6.6 % tertiary shoots respectively. The medium supplemented with BAP at 0.5 mg/L induced the highest number of shoots indirectly (3.5 ± 0.1 shoots/explant) while BAP at 1.5 mg/L induced the highest percentage of explant forming callus (84.4 ± 5.1 %). Therefore, double sterilisation method using Clorox and PPM at 1.25 µl/ml was optimum for surface sterilisation. A protocol for establishment of *C. nutans* culture is established.

Introduction

Clinacanthus nutans (Acanthaceae) is recognized as a valuable medicinal herb in Southeast Asia. The fresh leaves of *C. nutans* have long been used to treat venomous bites, varicella zoster virus infection, and skin rashes from measles in Thailand (Zulkipli *et al.*, 2017). Meanwhile, in Malaysia, the leaves of *C. nutans* are freshly eaten or blended with apple and consume as juice because of its nourishing and antioxidant properties (Shim *et al.*, 2013). The species has also been used as traditional remedy to treat sore throat, kidney problems, gout, prostate inflammation and skin problems like shingles (Yusmazura *et al.*, 2017). In Indonesia, the aqueous decoction of fresh leaves is prescribed to treat gastrointestinal problems, dysentery, dysuria, and a hyperglycemia (Hariana *et al.*, 2013; Zulkipli *et al.*, 2017). In China, different parts of *C. nutans* are consume on its own or in combination with other medicinal plants to treat ailments such as inflammation, strains,

rheumatism, anemia and jaundice (Farsi *et al.,* 2016; Zulkipli *et al.,* 2017). Currently, the cytotoxicity effects of *C. nutans* extract against human cancer cell line have gained numerous attention. This is because the extracts of *C. nutans* have been reported to have potent anticancer properties on non-small cell lung cancer (A549), nasopharygeal cancer (CNE1), and liver cancer (HepG2) (Ng *et al.,* 2017), breast cancer (MCF-7) (Teoh *et al.,* 2017) and human cervical cancer cell line (HeLa) (Yusmazura *et al.,* 2017). Despite the positive results reported by various researchers, particularly on the cytotoxic evaluation, the results were not consistent. The reason for this was because of the fluctuating levels of the active compounds in the raw materials. Production of secondary metabolites is influenced by the environmental condition such as elevation, rainfalls and temperature (Fong *et al.,* 2016).

In medicinal plants, plant tissue culture technology has been used to propagate, conserve, facilitates the improvement programme and to produce secondary metabolites. A prerequisite for the use of the technology is the establishment of the *in vitro* culture system for medicinal plants. The surface sterilization stage of explants is the most important step in a tissue culture protocol (Dodds and Roberts, 1985). Failure to clean the explants could lead to failure in establishing plants in cultures. The process of eliminating exogenous and endogenous pathogens in culture systems can be carried out using various materials including the use of hydrogen peroxide, mercuric chloride, sodium hypochlorite (Masafumi *et al.,* 2011; Wood *et al.,* 2011), fungicide (Haldeman *et al.,* 1987; Rihan *et al.,* 2012) and antibiotic treatment (Kneifel and Leonhardt, 1992; Leifert *et al.,* 1992; Rihan *et al.,* 2012). Although the use of these materials are reported to be effective for controlling and restraining contaminants, limitations still occur (Seckinger, 1995; Rihan *et al.,* 2012). Two essential limitations are the development of resistance by pathogens (Reed and Tanprasert, 1995; Rihan *et al.,* 2012) and exposure of explants to hazardous chemical such as mercuric chloride (Tiwari *et al.,* 2012). In this present study, Plant Preservatives Mixture (PPM) was used to reduce the gap between the limitations. PPM is a combination of two broad-spectrum industrial isothiazolone biocides, chloromethyl isothiazolone and methyliso-thiazolone (Rihan *et al.,* 2012). The effects of PPM on several species had been studied, among them were sweet orange, trifoliate orange, and rough lemon (Niedz, 1998); melon, petunia and tobacco (Compton and Koch, 2001); pepper and cauliflower (Rihan *et al.,* 2012).To date, the use of PPM on *C. nutans* has not yet been reported. Therefore, the objectives of this study were to optimize explant surface sterilisation method and determined the plant growth regulators for direct and indirect shoot regeneration from nodal explants of *C. nutans* from Malaysia.

Materials and Methods

Plant materials and surface sterilisation protocols

The donor plants of *C. nutans* were grown in the vicinity of Faculty of Science and Natural Resources, Universiti Malaysia Sabah. In this study, the stems of *C. nutans* were used as the source of explants.

Surface sterilisation methods of *ex vitroC. nutans*

The stems were washed thoroughly under running tap water for 30 minutes to remove soil debris. Then, the stems were cut into 3-4 cm length segments, each segment had at least one node.

Next, the nodal segments were soaked in

distilled water prior to surface sterilisation. The explants were sterilised using the methods as explained in Table (1).

Direct shoot multiplication

Sterilised nodal segments were trimmed to remove dead tissues at both ends before cultured on the media vertically. The culture media were made of full strength Murashige and Skoog (1962) basal medium supplemented with TDZ (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L), BAP or KN (0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75 and 2.0 mg/L) at different concentrations for single application, while for combination treatment of auxin-cytokinin, TDZ (0.1 mg/L) + NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L)or BAP (1.0 mg/L) + NAA (0.1, 0.2, 0.3, 0.4 and 0.5 mg/L) were evaluated. Sucrose (30 g/L) was added as the source of carbon, solidified with gel rite (4 g/L) and the pH was adjusted to 5.7. The mixture was autoclaved for 15 min at 121 ℃ and 1.05 kg/cm^2.MS medium without plant growth regulators was used as the control. The autoclaved media were poured into sterile glass jarseach containing 60 mL of medium.Each treatment was replicated 3 times with 10 explants for each replicate. The cultures were incubated at 16hours photoperiod at 25± 2 ℃.Shoot formation(primary, secondary and tertiary shoots) were observed and recorded after 60 days of incubation period.

Indirect shoot regeneration

Nodal segments (3-4 cm length) from *in vitro* grown plantlets of *C. nutans* were used as explants for indirect shoot regeneration study. The nodal segments were cultured horizontally on the shoot regeneration media. The shoot regeneration media consisted of full strength MS basal medium supplemented with either single treatment of BAP or KN at various concentrations (0, 0.25, 0.5, 1.0 and 1.5 mg/L), or combination treatment of thidiazuron (TDZ) at constant concentration of 0.1 mg/L with BAP or KN at various concentrations (0.25, 0.5, 1.0 and 1.5 mg/L).Sucrose (30 g/L) was added as the source of carbon, solidified with gelrite (4 g/L) and the pH was adjusted to 5.7. The mixture was autoclaved for 15 min at 121 ℃ and 1.05 kg/cm^2. MS medium without plant growth regulators was used as the control. The autoclaved media were poured into sterile Petri dishes with 25 mL of media in each dish. The cultures were then incubated in 16h photoperiod at 25 ℃ ± 2. Each treatment was replicated 3 times and each replicate contained 10 explants.

Rooting and acclimatisation

All regenerated shoots were transferred on semi-solid MS medium without plant regulators for leaf development and root induction for 30 days and subsequently acclimatised. Prior to acclimatisation, the length of the shoots and roots of the plantlets were measured. Each plantlet was acclimatized in plastic pots containing soil and coconut husk (1:1). The pots were covered with transparent polyethylene bags to reduce evaporation. The corners of the polyethylene bags were cut off after 3 days and completely removed after 2 weeks.

Data collection, formula and statistical analysis

All the experiments were carried out in factorial using a completely randomized design with 3 replicates. The means were analyzed statistically using IBM SPSS Statistics 24 (2017).The significance was determined by analysis of variance (ANOVA) and the least significant ($p \leq 0.05$) differences among mean values which were estimated using Duncan's Multiple Range Test. All the data obtained in this study were calculated by

using the formulas as stated below.

Percentage of explant survived =

$$\frac{\text{Total number of clean explants}}{\text{Total number of explants cultured}} \text{X } 100$$

Percentage of explants forming shoots =

$$\frac{\text{Total number of explant with shoots}}{\text{Total number of explants cultured}} \times 100$$

Number of shoots per explant =

$$\frac{\text{Total number of shoots}}{\text{Total number of explants formed shoots}}$$

Percentage of explant forming callus (or root)

$$= \frac{\text{Total number of explant formed callus (or root)}}{\text{Total number of explants cultured}} \times 100$$

Results and Discussion

Surface sterilisation methods of *ex vitro C. nutans*

In this study, various methods were tested to sterilise the explants with the goal to produce clean healthy cultures. Out of five (M1, M2, M2a M2b, M2c) methods tested, the single stage method (M1) had failed to eradicate contaminants on *C. nutans* (Figure 1), whereas, the double stage methods (M2, M2a, M2b, M2c) were effectivein cleaning the explants depending on the concentration of PPM used. Survival of explants was the highest (82 ± 4.5 %) when the washing solution contained 1.25 µl/ml PPM (M2a) as indicated on Figure 1. The lowest survival of explants was given by method M2 (27± 8.6 %) (Figure 1) which did not contain any PPM. There was 3 fold increase of explant survival between M2 and M2a methods, indicating the importance of PPM in the sterilizing step. However, when the concentration of PPM increased beyond 1.25 µl/ml, the percentage of cleaned explants reduced. This result is significant ($p \leq 0.05$) when comparing the concentration of PPM used in the double stage methods (M2, M2a, M2b, M2c) and method M1.

Direct shoot multiplications

In this study, various types and combinations of PGRs were tested. Shoots were formed on three locations on the explant; primary (1°), secondary (2°) and tertiary (3°) shoots (Figure 2B).Regardless of the type of treatments, all explants formed primary shoots at the nodes of the explants(Figure 2A).But the media supplemented with BAP at all concentrations (0.25, 0.5, 0.75, 1.0, 1.25, 1.50, 1.75, 2.0 mg/L) or KN at 0.75 and 1.0 mg/L did also produce additional secondary and tertiary shoots (Table 2). In the present study, the medium supplemented with 1.5 mg/L BAP formed the highest number of primary (4.4±2.4), secondary (1.3±0.1) and tertiary (1.2±0.3) shoots per explant, respectively as indicated in Table 2. Meanwhile, the media supplemented with combination treatment of PGRs produced lower number of shoots as compared to the single application of PGR. Treatment with combined PGRs (all treatment) only produced primary shoots with the highest was given by 1.0 mg/L BAP + 0.3 mg/L NAA (3.4± 0.4 shoot/explant). While, the lowest was recorded by 0.1 mg/L TDZ + 0.4 mg/L NAA (1.0 ± 0.5 shoots/explant). Table 2 shows the respond of explants on shoot regeneration media supplemented with PGR.

Indirect shoot regeneration

The nodal explants were cultured horizontally to observe the effects of single treatment of BAP and KN on shoot regeneration. In this approach, calli were formed prior to shoots regeneration, followed by roots formation (Figure 2C and 2D). For the formation of callus, all media successfully induced callus

on explants, but 1.5 mg/L BAP resulted in significantly ($p \leq 0.05$) higher percentage of explant forming callus (84.4%) as indicated in Table 2. Meanwhile, the medium supplemented with 0.25 mg/L KN and the control medium failed to induce callus.

The highest number of shoots regenerated per explant was on the medium supplemented with 0.5 mg/L BAP, which produced 3.45±0.1 shoots. The lowest was produced by the treatment with 1.5 mg/L KN, which recorded 1.3±0.1 shoots. As for the formation of roots, the highest percentage of explant forming roots was those explants on the control medium (86.4 ±3.2 %). While 0.5 mg/L BAP resulted in the lowest explant forming roots (1.33±1.3 %).

Addition of 0.1 mg/L TDZ into the media containing BAP (0.25, 0.50, 1.0, and 1.5 mg/L) had reduced the ability of the explants to form callus and shoots (Table 3). Between 1.9-7.6 fold decrease in callus formation and between 0.7-1.3 fold reductions in shoot formation were observed when the media were supplemented with TDZ. In contrast, addition of TDZ into the media with KN did increase the capability of the explant forming callus and shoots, however the increment was not significant. The presence of TDZ in the media containing KN also inhibited the formation of roots by the explants, except for the medium supplemented with 0.25 mg/L KN which resulted in 5.32±1.5 % explant to form roots.

Rooting and acclimatisation

After shoots were regenerated, the *in vitro* plantlets were transferred on MS medium without plant growth regulator (MSO) for root induction and shoot development. After 4 weeks, the regenerated shoots elongate with the average length of 3.4±0.4 cm (Table 5) while the average length of roots were3.8±0.4 cm which were suitable for acclimatisation (Table 4). After 2 weeks of acclimatisation in natural light condition, 100% of plantlets survived (Figure 2E).

Table.1 Surface sterilization protocols for *Clinacanthus nutans* nodal segments

No.	Methods	Treatment Code	Sterilisation Procedure	References	Remarks
1	Method 1	M	The nodal segments were soaked in 70 % (v/v) ethanol for 10 seconds followed by soaking in 20 % (v/v) Clorox + Tween 20 (1 drop) at pH 6 for 30 minutes. Finally, the stems were rinsed three times using sterilised distilled water.	Webster *et al.*, (2003)	Single sterilisation method
2	Method 2, Method 2a, Method 2b, Method 2c	M2 M2a M2b M2c	Similar procedure as in Method 1 was applied on the first stage, but the rinsing was replaced with soaking in sterilised distilled water for 10 minutes. For the second stage, the explants were further treated by soaking in 10 % (v/v) Clorox + Tween 20 (1 drop) for 10 minutes. Finally, the nodal segments were rinsed with Plant Preservatives Mixture (PPM) in sterilized distilled water at different concentrations which were denoted asM2 (0 PPM), M2a (1.25 µl/ml PPM), M2b (2.50 µl/ml PPM), and M2c (5.00 µl/ml PPM).		Double sterilisation method

Table.2 The effects of various concentrations of PGR on a percentage of shoot formed

Plant growth Regulators (mg/L)	Explant forming primary shoots (%)	Number of primary shoots per explant (mean ± S.D.)	Explant forming secondary shoots (%)	Number of secondary shoots per explant (mean ± S.D.)	Explant forming tertiary shoots (%)	Number of tertiary shoots per explant (mean ± S.D.)
CONTROL 0	100	2.2 ± 0.8	0	0	0	0
BAP 0.25	100	2.7 ± 1.1	100	1.0 ± 0	0	0
BAP 0.5	100	3.0 ± 1.0	100	1.0 ± 0	100	1.0 ± 0
BAP 0.75	100	3.4 ± 1.5	100	1.3 ± 0.3	100	1.0 ± 0
BAP 1.0	100	3.8 ± 1.3	100	1.3 ± 0.2	100	1.0 ± 0
BAP 1.25	100	4.0 ± 1.4	100	1.3 ± 0.2	100	1.2 ± 0.3
BAP 1.5	**100**	**4.4 ± 2.4**	**100**	**1.3 ± 0.1**	**100**	**1.2 ± 0.3**
BAP 1.75	100	4.3 ± 1.8	100	1.2 ± 0.2	100	1.2 ±0.3
BAP 2.0	100	3.8 ± 1.6	100	1.0 ± 0	100	0
KN 0.25	100	2.2 ± 0.9	0	0	0	0
KN 0.5	100	2.4 ± 0.8	0	0	0	0
KN 0.75	100	2.4 ± 0.7	100	1.0 ± 0	0	0
KN 1.0	100	2.6 ± 0.7	100	1.0 ± 0	0	0
KN 1.25	100	2.4 ± 0.6	0	0	0	0
KN 1.5	100	2.4 ± 0.7	0	0	0	0
KN 1.75	100	2.3 ± 0.7	0	0	0	0
KN 2.00	100	2.3 ± 0.6	0	0	0	0
TDZ 0.1	100	3.3 ± 1.4	0	0	0	0
TDZ 0.2	100	3.0 ± 0.8	0	0	0	0
TDZ 0.3	100	3.8 ± 2.0	0	0	0	0
TDZ 0.4	100	1.6 ± 0.8	0	0	0	0
TDZ 0.5	100	1.2 ± 0.4	0	0	0	0
BAP 1.0 + NAA 0.1	100	1.8 ± 0.5	0	0	0	0
BAP 1.0 + NAA 0.2	100	1.9 ± 0.5	0	0	0	0
BAP 1.0 + NAA 0.3	100	**3.4 ± 0.4**	0	0	0	0
BAP 1.0 + NAA 0.4	100	2.1 ± 0.8	0	0	0	0
BAP 1.0 + NAA 0.5	100	1.8 ± 0.3	0	0	0	0
TDZ 0.1 + NAA 0.1	100	2.2 ± 0.7	0	0	0	0
TDZ 0.1 + NAA 0.2	100	2.1 ± 0.7	0	0	0	0
TDZ 0.1 + NAA 0.3	100	1.6 ± 0.7	0	0	0	0
TDZ 0.1 + NAA 0.4	100	1.0 ± 0.5	0	0	0	0
TDZ 0.1 + NAA 0.5	100	1.2 ± 0.4	0	0	0	0

Percentage of primary/secondary/tertiary shoot formed represent three replicates per treatment. Values in each column are number of primary/secondary/tertiary shoots per explant ± standard deviation (SD) represent three replicates per treatment (n=30).

Table.3 The effect of the application of BAP, KN alone and in combination (with a constant concentration of TZD at 0.1 mg/l) on callus formation, shoots regeneration and root formation of *C. nutans*

Plant growth Regulators (mg/L)			Explant forming callus (% ± S.D.)	Number of shoots formed per explant (mean ± S.D.)	Explant forming root (% ± S.D.)
Control	0		0 ^{f}	2.27± 0.1^{e}	**86.4± 3.2 a**
KN	**BAP**	**TDZ**			
0.25	0	0	0^{f}	1.71± 0.1^{e}	9.97± 1.5 ^{e}
0.5	0	0	1.11± 1.9 ^{ef}	1.81± 0.1^{e}	15.2± 1.5 ^{d}
1.0	0	0	36.7± 3.3 ^{d}	2.6± 0.1^{c}	1.3± 1.3 ^{g}
1.5	0	0	7.7± 1.9 ^{e}	1.3± 0.1^{f}	18.2± 1.5 ^{c}
0	0.25	0	58.9± 1.9 ^{b}	2.07± 0.1^{d}	25.2± 3.3 ^{b}
0	0.5	0	44.4± 7.7 ^{c}	**3.45± 0.1^{a}**	1.3± 1.3 ^{g}
0	1.0	0	54.4± 1.9 ^{b}	2.16± 0.2^{d}	16.1± 1.6 ^{cd}
0	1.5	0	**84.4± 5.1 ^{a}**	3.07± 0.1^{b}	1.3± 1.2 ^{g}
0	0	0	0 ^{f}	0^{g}	0 ^{g}
0.25	0	0.1	44.4± 5.1 ^{c}	2.13± 0.1^{d}	5.3± 1.5 ^{f}
0.5	0	0.1	33.3± 6.7 ^{d}	1.86± 0.1^{e}	0 ^{g}
1.0	0	0.1	35.6± 1.9 ^{d}	**2.16± 0.1^{d}**	0 ^{g}
1.5	0	0.1	33.3± 6.6 ^{d}	2.49± 0.1^{c}	0 ^{g}
0	0.25	0.1	7.77± 1.9 ^{e}	2.26± 0.1^{d}	0 ^{g}
0	0.5	0.1	7.72± 3.9 ^{e}	2.61± 0.1^{c}	0 ^{g}
0	1.0	0.1	60.0± 3.3 ^{b}	**3.13± 0.1^{b}**	0 ^{g}
0	1.5	0.1	45.6±1.9 ^{c}	2.67± 0.1^{c}	0 ^{g}

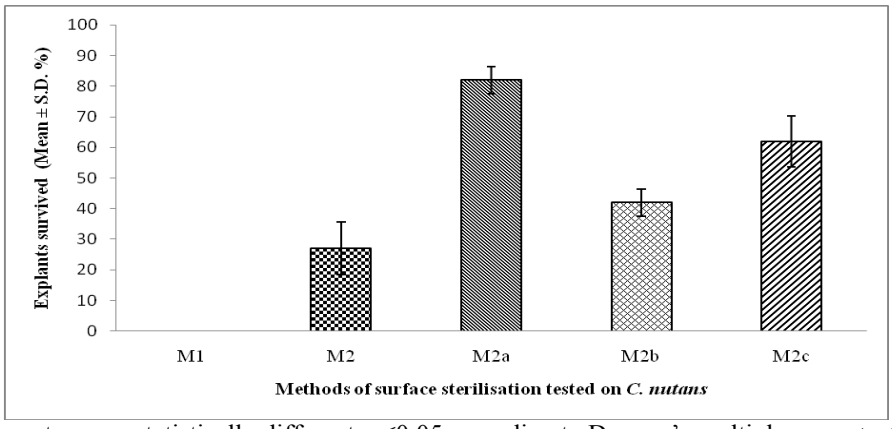
Data recorded on the 60th day and represent a total of 90 explants per treatment. Different lowercase letters within columns indicate that these means of percentage are statistically different at $p \leq 0.05$ according to Duncan's multiple range tests.

Table.4 The length of shoots and roots after 4 weeks transferred on MS medium without plant regulator

Rooting media	Total number of plantlets	Length of shoot (cm ± S.D)	Length of root (cm ± S.D)	Plantlet Acclimatised (%)
MSO	30	3.4 ± 0.4	3.8 ± 0.4	100

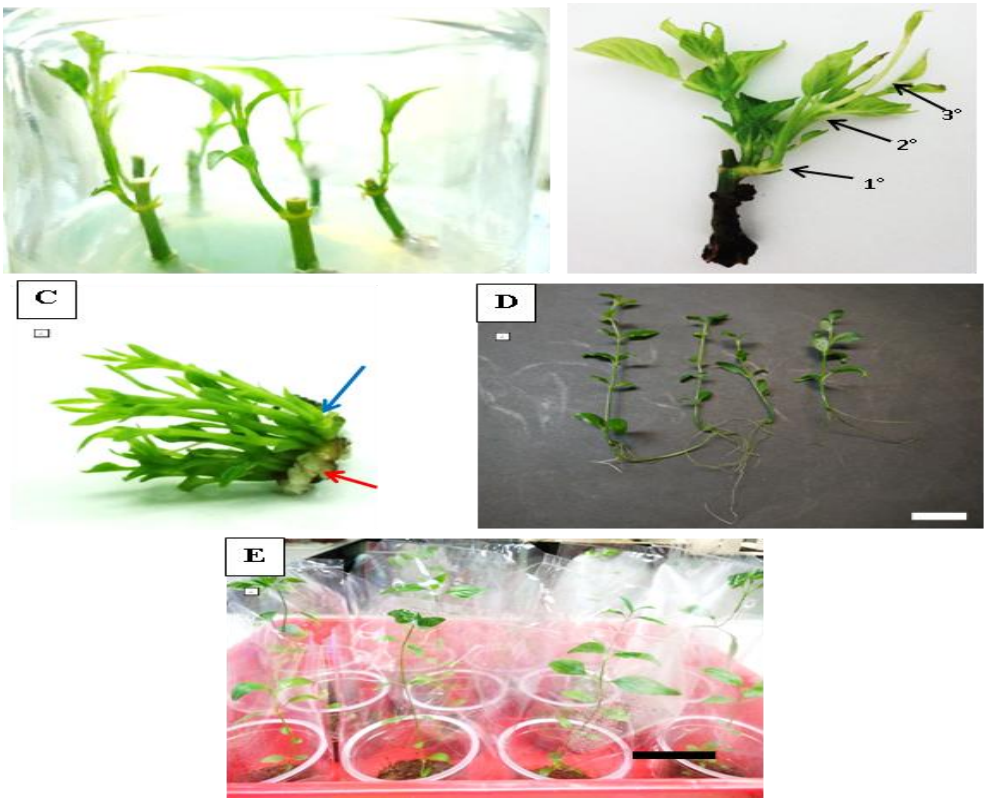
(n=30). The plantlets that survived the acclimatisation process were recorded after four weeks.

Figure.1 The effects of various surface sterilisation methods on the survival of nodal segments of *C. nutans*



The means of percentage are statistically different, p≤0.05 according to Duncan's multiple range tests. The error bars on the top of the bar as standard deviations for the treatments.

Figure.2 Establishment of C. *nutans* culture. **(A)** Explant that survived the surface sterilization using Method 2b (Bar = 1.2 cm); **(B)** Multiple shoots generated on explants cultured vertically - primary (1°), secondary (2°), and tertiary (3°) shoots (Bar = 1.0 cm); **(C)** Shoot regenerated indirectly on explant cultured vertically (Red arrow: callus formed; Blue arrow: shoots regenerated from callus) (Bar = 0.5 cm); **(D)** Roots developed on plantlets cultured on MS0 medium (Bar = 1.0 cm); **(E)** Acclimatised *C. nutans* (Bar = 1.25 cm)



Establishment of surface sterilisation methods on *C. nutans*

The usage of ethanol 70% (v/v), disinfectant and surfactant in single and double stages for surface sterilisation process plays a vital role in providing an effective surface sterilisation method. The present study shows that double sterilisation methods (M2, M2a, M2b, and M2c) are effective against microorganisms on *C. nutans*. This was supported by Tiwari *et al.,* (2012) working on sugarcane, which stated that the usage of disinfectant in one-step sterilization method was less effective as compared to two-step sterilisation protocols. This recent study also proved that the rinsing process plays an important role in the sterilisation process. Addition of PPM in the rinsing water was effective in eradicating contaminants on *C. nutans* nodal cuttings (M2a, M2b, and M2c). However, PPM had an inverse interaction between concentration and the survival rate of explants due to its toxicity. This is also supported by the findings of Compton and Koch (2001) whereby the concentration of PPM above 5 mL/L had caused negative impacts on the formation callus by the cotyledon explants of melons. The inhibitory effect of PPM on shoot regeneration of chrysanthemum was also reported by George and Tripepi, (2001). Their work showed that 2 mL/L PPM reduced the number of viable explants. In contrast, a study by Niedz (1998) indicated that PPM had positive effects on sweet orange (*C. sinensis L.*), viability – the viability of explants was higher when the concentration of PPM used was lower than 1.0 mL/L.

Plant Preservative Mixture controls the growth of microbes by penetrating the microbial cell wall thus inhibiting several key enzymes in the citric acid cycle, this in turn disrupting the electron transport chain and inhibit the transport of monosaccharide and amino acids from media into microbial cells (George and Tripepi, 2001). Due to the complexity of plant cell wall, PPM molecules are reported not to be affecting the metabolic and transport pathways of plant tissues (George and Tripepi, 2001; Plant Cell Technology, 2019). However, in the present study, death of explants was observed in the double sterilisation methods (M2, M2a, M2b, and M2c) with increasing concentration of PPM. Thus, indicating that the concentration of PPM molecules can influence the metabolic and transport pathways of *C. nutans* tissues. Hence, the effective volume of PPM should be tested on desired plants prior to establishing sterilisation protocols. Nevertheless, double sterilisation in this study is effective as an alternative method to eradicate contaminants.

Direct shoot multiplication

Plant growth regulators (PGR) are required in relatively low quantity in plant tissue cultures as compared to *ex vitro* cultures.This requirement variesaccording to the level of endogenous hormones of the explants (Loyola-Vargas and Ochoa-Alejo, 2012).The use of single cytokinin and cytokinin-auxin in the current study were purposely done to induce multiple shoots as PGRs play important role during organogenesis.

The current work shows that BAP alone was effective in inducing optimal direct shoot multiplication as compared to KN and TDZ supplemented alone. BAP was able to induce shoot at all three levels. This is because BAP stimulates growth by inducing plant cell division. According to Buah *et al.,* (2010) BAP is stability retained in *in vitro* cultures, this is because BAP is not easily broken down. Therefore, plant tissuesreadily absorbed BAP either in free or ionised forms and subsequently promotes shoot formation. The current results is in agreement with the investigation done by Namli *et al.,* (2010) on

Hypericum retusum, and Khalekuzamman *et al.,* (2008) on *Adhatodavasica* Nees. In those previous works the media supplemented with BAP alone were able to produce the highest number of shoots compared to that supplemented with other cytokinin.

KN supplemented alone performed poorly in inducing direct shoot multiplication as compared to BAP. Comparable findings regarding KN having low effectiveness as compared to BAP were reported on *Aegle marmelos* (Namli *et al.,* 2010) and *Coleonema album* (Fajinmi *et al.,* 2014). Similar to KN, TDZ had no shoot promotional effect on *C. nutans*. At the optimal concentration of TDZ, the number of primary shoots formed per explant was higher as compared to control medium (without PGR) and the media with KN alone (Table 2). In the present study, TDZ was relatively effective in inducing shoot at lower concentration compared to BAP and KN. In tissue culture, TZD promote proliferation of multiple shoot but at high concentration it can cause the formation of shorter shoot, suggesting an inhibition effect on elongation (Parveen and Shahzad, 2010). For the present study, the inhibitory effect of TDZ could be associated to the herbicidal properties of the PGR.

Indirect shoot regeneration

The orientation of the explants on the medium had affected the nature of regeneration of *C. nutans*. Callus was formed at the cut ends of the explant segments due to the constant contact of the explant to the medium during incubation period (Arockiasamy *et al.,* 2002; Sharma and Wakhlu, 2010; Bhusare *et al.,* 2018). Based on the present results, BAP alone was effective in inducing both callus and shoot regeneration as compared to KN alone and the control medium.

Addition of TDZ (0.1 mg/L) into the media containing KN and BAP (Table 3) at various concentrations did not enhance indirect shoot regeneration. However, TDZ (0.1 mg/L) together with BAP at a 1.0 mg/L was slightly better in inducing shoot (3.13 ±0.1 shoot per explant) as compared to TDZ with KN at 1.0 mg/L(2.16±0.1 shoot per explant). This study is in coherent with the study by Kakar *et al.,* (2014) on *Brassicarapa var. turnip* which showed moderate response of shoot number under the influence of TDZ and KN as compared to TDZ and BA.

This study concludes, an effective surface sterilisation method for *C. nutans* from Sabah, Malaysia was achieved through double sterilization method using Clorox and PPM (1.25 mL/L) in the final rinsing. Multiplication of shoots was best obtained on MS medium supplemented with 1.5mg/L BAP which produced 4.4±2.4, 1.3±0.1 and 1.2±0.3primary, secondary and tertiary shoots per explant respectively. Indirect shoot regeneration of *C. nutans* was optimum on the medium containing 0.5 mg/L BAP, which had resulted in 3.45±0.1 shoot regenerated.

Conflict of interest statement

We declare that we have no conflict of interest.

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