ISSN (E): 2320-3862
ISSN (P): 2394-0530
https://www.plantsjournal.com
JMPS 2023; 11(5): 36-46

Received: 10-07-2023
Accepted: 13-08-2023

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Effects of various plant growth regulators upon callus induction potential using leaf explants of *Clinacanthus nutans* (Sabah Snake grass)

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DOI: https://doi.org/10.22271/plants.2023.v11.i5a.1585

Abstract
Sabah snake grass, also known as *Clinacanthus nutans*, is a medicinal plant which has been widely consumed by natives in the Asian countries to treat many ailments**.** At present, stem cutting is the only way this plant is propagated which in future could lead to extinction. Besides, variation in the active compounds had been reported in fresh samples of *C. nutans*. Not many *in vitro* works had been carried out to cultivate this plant. The aim of this study was to establish clean (*in vitro*) cultures of *C. nutans* and determine the optimal callus induction and proliferation protocol, using various combination of plant growth regulators at different concentrations, ranging from 0-2.0mg/l, using leaf-segments. Highest percentage (87.88%) of aseptic *C. nutans* shoot cultures were successfully obtained from nodal segments disinfected using in 50% home bleach Clorox® for 20 minutes, and with 30% Clorox® for 50 minutes. Best callus induction was obtained from explants cultured on Murashige and Skoog media supplemented with Picloram alone at 0.5mg/l. This protocol would be useful to minimize the destruction of wild grown *C. nutans* and effective callus propagation would be beneficial to growing resources in a big scale.

Keywords: Callus, *Clinacanthus nutans*, *in vitro*, leaf explants, plant growth regulators

Introduction
Medicinal plant can be defined as plants that contain valuable compounds with therapeutic effects in healing and preventing various ailments (Fridlender *et al*. 2015) [1]. Currently, more than half of the world population, both in developed and developing countries consume herbal plants as health concoction for the prevention and treatment of various illnesses (Abera 2014) [2]. The valuable plant-based compounds are sought after by pharmaceuticals, cosmetics, and nutraceuticals industries owing to their affordability, accessibility and being safer than their counterparts, the synthetic chemicals. Besides, consumers have become more attentive about their current lifestyle which had spiked the requirement for natural compounds for consumption. One of the valuable medicinal plants that has been in demand in the market laytely is *Clinacanthus nutans*. This plant is a tropical medicinal plant widely used by the natives in Asian countries such as Malaysia, Thailand, and Indonesia. Locally known as the "Sabah snake grass," or Daun Belalai Gajah, it is a perennial shrub with oblong leaves, growing on opposite sides of the waxy stems that thrives in semi-shaded areas with sandy clay soil (Ismail *et al*., 2017) [3]. The leaves are traditionally used to treat diabetic, blood pressure (Pannangtech 2007) [4], skin rashes (Sakdarat *et al*., 2006) [5], snake bites (Daduang *et al*., 2005) [6] and insect stings (Uawonggul *et al*., 1978) [7]. Besides, pharmacological properties such as anti-inflammatory (Wanikat *et al*., 2008) [8], anti-*Herpes Simplex virus* (Kunsom *et al*., 2013) [9], anti-*Varicella Zoster* virus (Charuwichitaratana *et al*., 1996) [10], anti-proliferative (Yong *et al*., 2013; Sulaiman *et al*., 2013) [11-12], anti tumor (Huang *et al*., 2015; Widjaja *et al*., 2021) [13-14] and anti-cancer properties (Fong *et al*., 2016; Yuzmazura *et al*., 2017) [15-16] has been reported. This is due to its richness in its secondary compounds such as stigmasterol (Damphawan, 1976) [17], lupeol (Tu *et al*., 2014) [18], vitexin and orientin (Teshima *et al*., 1997) [19] and sulphur containing glucosides (Satakhun, 2001) [20]. Though fresh and dried leaves of *C. nutans* have been consumed by locals to treat various ailments, variation in its active compounds have been stressed. Age of harvesting (Ghasemzadeh *et al*., 2014) [21], storage duration of leaves (Raya *et al*., 2015) [22], drying method of the leaves (Abdullah and Aziz, 2018) [23] and different soil conditions (Ismail *et al*., 2017) [3] had exhibited dissimilarities in the content of active compounds of *C. nutans*.

Hence, the change in phytochemical constituents present in herbal plants due to climate change and different soil component and composition highlights the need for proper standardizing procedures which is crucial for preservation and upgrading the content of any medicinal plant. As such, enhancing the active compounds content in *C. nutans* under *in vitro* conditions would allow consistent levels and type of phytochemical production, with the absence of ecological pressure. Micropropagation could help to obtain a uniformity in its content that are not dependent to seasonal demand. There are not many reported micropropagation or tissue culture studies in *C. nutans*, hence this study was initiated (Bong *et al*. 2021; Haida *et al.* 2020, Chen *et al.* 2015) [24-26] with a proper protocol for establishment of clean cultures.

Callus refers to proliferation of dedifferentiated cells (Feher, 2019) [27]. Induction of callus is seen as a tool in plant biotechnology, especially for genetic manipulation of plants (at a cellular level), micropropagation and study of plant metabolism. Currently, with the emphasis on natural products from plants, callus is identified to be useful towards commercial production of these naturally occurring plant metabolites using callus derived suspension cell cultures (Hussain *et al*., 2012) [28]. To obtain the desired callus formation (soft, friable, hard or brown) it depends on the variation of plant growth regulators added (Ikeuchi *et al*. 2013) [29]. Establishment of callus can be the stepping stone to produce desired secondary metabolites from valuable medicinal plants using cell suspension culture as this could provide an alternative method to traditional whole plant cultivation (Li and Tao, 2009; Baldi and Dixit, 2008; Jeong *et al*., 2008) [30-32]. However, limited study had been carried out on callus induction in *C. nutans* (Bong *et al*., 2021; Hashim *et al*., 2021 and Phua *et al*., 2016) [24, 33-34] whereby callus study was carried out using limited plant growth regulators (2,4-D,Kinetin and BAP). To date, no callus study had been initiated using Picloram in *C. nutans* though many callus induction studies using Picloram had been reported in other medicinal plants (Gnasekaran *et al*., 2023; Gantait and Mahanta 2021; Suwanseere *et al*., 2019; Rahayu *et al*., 2016) [35-38]. This is first study in *C. nutans* to induce callus using Picloram as one of the plant growth regulators. Hence, in this study, callus induction in *C. nutans* was carried out using different auxins and cytokinins, at different concentrations. This study will further aid to produce bioactive compounds from *C. nutans* using callus-derived suspension cell cultures in bioreactors, paving way for a possible scale-up studies.

Materials and Methods

Materials: Benomyl fungicide; Home bleach (Clorox®); Ethanol (AR grade); NAA (1-Napthaleneacetic acid) (Duchefa, Germany), 2, 4-D (2, 4-Dichlorophenoxyacetic acid) (Sigma Aldrich); Picloram (4-amino-3, 5, 6-trichloropicolinic acid) (Sigma Aldrich; BAP(6-Benzylamino purine) (Duchefa, Germany);Kinetin (6-Furfuryl-aminopurine) (Sigma Aldrich); Murashige and Skoog salt and vitamins (MS) (Duchefa, Germany); Gelrite (Duchefa, Germany); Sucrose (Chem Soln).

Surface sterilization of *C. nutans* explants

The mother plant *Clinacanthus nutans* used in this research was obtained from the Botany Department, Forest Research Institute Malaysia (FRIM), Kepong, Selangor and authenticated and deposited at the herbarium in FRIM, with the voucher no PID 111121-08. Nodal segments of *C. nutans* (approximately 2-3cm) in length were first, washed in running tap water for an hour, followed by soaking in 0.1% (w/v) benomyl fungicide for an hour, and then rinsed several times with sterilised distilled water (SDW). Next, a three-step surface sterilization protocol was used, where, first, soaking in 70% ethanol for 1 minute, followed by agitating in 50% domestic Clorox ® (v/v) (containing 5.2% sodium hypochlorite) with two drops of Tween-20, at 3-different exposure time (i.e., 20, 30, 40 minutes), then again agitated in 30% domestic Clorox® (v/v) for 3-different exposure time (i.e. 20, 40 and 50 minutes). After each Clorox® treatment (Table 1) the explants were rinsed at least 3 - 4 times with SDW, or until all traces of detergent were gone. Surface sterilized explants were then blot dried on sterile tissue towels, in a laminar air flow cabinet, trimmed to remove all bleached tissue, to about 1.0 to 1.5 cm, before culturing onto basal Murashige and Skoog (MS) media, and incubated under ambient culture condition (25 ± 2 ℃, 12-hour photoperiod, 50-60% Relative Humidity (RH), in growth rooms. Cultures were observed weekly for signs of contamination, and clean emerging shoots were dissected and subcultured on to fresh basal MS media, to encourage further shoot development. Explant survival rates were estimated after 30 days of incubation, using the following formula, i.e.

$$\text{Survival (\%)} = \frac{\text{Number of explants survived}}{\text{Total of explants cultured.}} \text{ x } 100$$

Table 1: Different concentration and exposure time of Clorox® as part of triple stage sterilization protocol

Treatment	70% Ethanol	50% Clorox	30% Clorox
Method 1	1 min	30 min	40 min
Method 2	1 min	40 min	20 min
Method 3	1min	20 min	50 min

Callus induction of *in vitro C. nutans*

The effect of different growth regulators and its concentration on callus induction were determined using young leaves of *C. nutans* leaves. Young leaves were selected based on a preliminary study which was carried out using various explants to induced callus in *C. nutans*. In a five-by-five factorial design, MS media containing various plant growth regulators (PGRs) was combined at different concentrations ranging from 0.0 mg/l to 2.0 mg/l and poured into sterile, disposable petri dishes. MS media was prepared using 4.4g/l MS mineral salts, 3% sucrose and 0.3% Gel rite agar. The media pH adjusted to 5.6±0.1 (prior to adding of gelling agent) by using 1 molar HCL or NaOH. Media was autoclaved at 121 ºC under pressure (1.06 kg/cm2) for 15 minutes. The experiment was carried out in a completely randomized design (CRD). Four leaf cuttings of approximately 0.5 by 0.5 cm in size were placed on each sterile media. Each treatment was replicated 2 times. The cultures were maintained at 25 ± 2 °C and 60-65% relative humidity, at 12-hours photoperiod, with light intensity at 3000lux. Observation such as callus formations, calli texture, color and friability were recorded at the end of week-3 and week-6. The obtained calli were subcultured at the end-of week-8.

Percentage of callus induction in week-3 and week-6 was calculated using the formula as per below:

$$\text{Callus induction (\%)} = \frac{\text{Number of explants formed callus}}{\text{Total number of explants cultured.}} \text{ x } 100\%$$

Optimized callus induction media

The best combination of media from each factorial experiment carried out above was then selected and used further to determine the highest biomass induction media. This was based on the highest proliferation rate obtained, texture and color of the callus produced. The young leaves were cut into four and placed in the five best MS media supplemented with PGRs to induce calli. Two replicates with a total of 8 explants were cultured for each treatment. The most appropriate media for callus induction was determined by recording the fresh callus weighed at every subculture, that is week-8 (1st subculture), week-16 (2nd subculture) and week-24 (3rd subculture) from the date of initiation. The percentage of increase in biomass was calculated based on the change in biomass between 2nd and 3rd subculture. Besides, biomass (g), readings such as color and texture of calli were also noted. The best callus induction medium for *C. nutans* was determined based on the increase in fresh weight (biomass), along with the desired morphology such as friable and creamy callus.

Percentage of increase in fresh biomass was calculated using the formula as per below:

$$\text{Increase in Fresh Biomass (\%)} = \frac{\text{Biomass FW3 (g)} - \text{Biomass FW2 (g)}}{\text{Biomass FW2(g)}} \times 100$$

The data obtained were analyzed using SPSS version 25.0 software and Analysis of variance test at 95% confidence interval. The value of $P < 0.05$ was considered statistically significant.

Results

Sterilization of nodal cutting of *C. nutans*

The cultured nodal segments of *C. nutans* using different sterilization techniques produced over 70% clean cultures, for all the three (3) different sterilization methods tested (Table 2). In method one, nodal cuttings exposed to 50% Clorox® for 30 minutes and 30% Clorox for 40 minutes gave 71.43% of viable explants compared to method two of which the nodal cuttings exposed to 50% Clorox for 40 minutes and 30% Clorox for 20 minutes gave 72.22% of viable explants. However, method three, which was immersing of nodal cuttings of *C. nutans* in 50% Clorox for 20 minutes (shorter initial exposure time) and 30% Clorox for 50 minutes (longer later exposure time) gave the highest percentage of contaminant free cultures (over 87%). Method one and method two exhibited a high percentage of non -viable explants with 17.14% and 19.44% respectively. Contamination was judged visually and it was detected as early as day-2 of culture (Figure 1). Browning of explants leading to tissue death was evident at higher initial concentration of disinfectant.

Table 2: Percentage of viable, contaminated, and dead nodal explants of *C. nutans* decontaminated using different concentration of Clorox® at varying exposure time, after 4 weeks

	Treatment			Observation		
	Clorox 50%	**Clorox 30%**	**SMP**	**Viable explants (%)**	**Contaminated (%)**	**Dead (%)**
M1	30 min	40 min	35	71.43	11.43	17.14
M2	40 min	20 min	36	72.22	8.33	19.44
M3	20 min	50 min	33	87.88	9.09	3.03

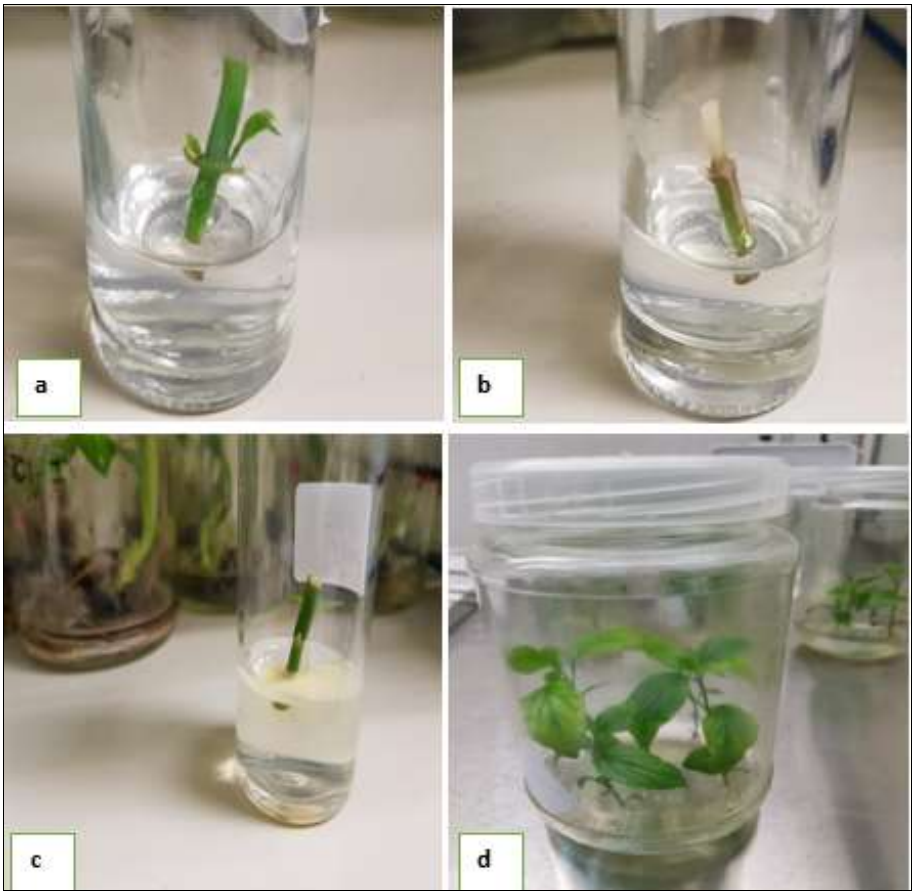

Fig 1: (a) Young regenerant of *C. nutans* shoot from nodal segments at week-2 of culture; (b) Dead nodal explant due to sterilant effect; (c) Bacteria contaminant of explant, seen day 2 of culture; (d) Complete *C. nutans* plant, after 6-weeks in culture media.

Effect of plant growth regulators on callus induction from leaf explants of *C. nutans*

Visible callus initiation on the leaf cuttings were seen after six weeks of initiation. Depending on the concentration and type of plant growth regulators (PGR) used, level of callus formation and morphological characteristics varied. The percentage of callus formed refers to the number of explants that formed callus according to the proliferation rate, with one plus referring to a small portion of callus induced on the cut leaf, two plus referring to half of the cut leaf forming callus, three plus refers to three quarter of leaf formed callus and four plus refers to the callus formed covering the whole cut leaf. MS media without plant growth regulators (Control) failed to induce callus using *C. nutans* leaves. From the various treatments using different auxins (2, 4-D, NAA and Picloram) and cytokinin (BAP and Kinetin), it is observed the presence of cytokinin alone did not induce any calli formation. However, MS media supplemented with either auxin alone (Picloram/NAA) or in combination with cytokinin were able to induce calli formation.

Effect of NAA with the presence of BAP or Kinetin in inducing callus

In MS+NAA+BAP with concentration ranging from 0.0mg/l - 2.0mg/l, brownish and hard textured calli were formed (Table 3). The presences of NAA alone produced small amount of calli (25%) at low concentration and gradually increased at higher concentration (100%). The addition of BAP gave rise to an increased percentage of callus formation, but the amount formed were small. A much lower success rate of callus formation was observed in MS media supplemented with NAA and Kin based on the factorial treatment (Table 4). Lower concentration of Kin produced brownish and soft calli compared to hard, brown and compact calli at higher concentration of Kin. Highest callus induction was recorded in MS+NAA (0.25 mg/l) + Kin (1.0 mg/l).

Table 3: Percentage of callus formation on MS media supplemented with NAA (0-2.0 mg/L) and BAP (0-2.0 mg/L), after six weeks of culture

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0 NAA	0%		
0.25 NAA	50% (+); 50% (++)	Brown	Hard
0.5 NAA	25% (+); 75% (++)	Brown	Hard
1.0 NAA	75% (+++);25% (++++)	Brown	Hard
2.0 NAA	100% (++++)	Brown	Hard
0 BAP	0%		
0.25 BAP	25% (+)	Brown	Hard
0.5 BAP	100% (+)	Brown	Hard
1.0 BAP	100% (+)	Brown	Hard
2.0 BAP	50% (+); 50% (++)	Brown	Hard
0.25 NAA+0.25 BAP	0%		
0.25 NAA+0.5 BAP	100% (+)	Brown	Hard
0.25 NAA+1.0 BAP	100% (+)	Brown	Hard
0.25 NAA+2.0 BAP	100% (+)	Brown	Hard
0.5 NAA+0.25 BAP	25% (+); 75% (++)	Brown	Hard
0.5 NAA+0.5 BAP	100% (+)	Brown	Hard
0.5 NAA+1.0 BAP	100% (+)	Brown	Hard
0.5 NAA+2.0BAP	100% (+)	Brown	Hard
1.0 NAA+0.25 BAP	50% (++); 25% (+++); 25% (+++++)	Brown	Hard
1.0 NAA+0.5 BAP	50% (+); 37.5% (++);12.5% (+++)	Brown	Hard
1.0 NAA+1.0 BAP	12.5% (+); 87.5% (++)	Brown	Hard
1.0 NAA+2.0 BAP	100% (+)	Brown	Hard
2.0NAA+0.25 BAP	50% (+); 37.5% (++); 12.5% (+++)	Brown	Hard
2.0 NAA+0.5 BAP	50% (+)	Brown	Hard
2.0 NAA+1.0 BAP	50% (++)	Brown	Hard
2.0 NAA+2.0 BAP	100% (+)	Brown	Hard

+ small spot of calli, ++ half explants covered with calli, +++ three quarter of explant covered with calli, ++++ fully covered with calli

Table 4: Percentage of callus formation on MS media supplemented with NAA (0-2.0 mg/L) and Kinetin (0-2.0 mg/L), after six weeks of culture.

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0.0 NAA	0%		
0.25 NAA	62.5% (+); 37.5% (++)	Brown	Hard
0.5 NAA	50% (+)	Brown	Hard
1.0 NAA	12.5% (+++); 75% (++); 12.5% (+)	Brown	Hard
2.0 NAA	100% (++)	Brown	Hard
0 Kin	0%		
0.25 Kin	0%		
0.5 Kin	0%		
1.0 Kin	0%		
2.0 Kin	50% (+)	Brown	Hard
0.25 NAA +0.25 Kin	87.5% (+)	Brown	Hard
0.25 NAA + 0.5 Kin	50% (+)	Brown	Soft
0.25 NAA + 1.0 Kin	100% (++++)	Brown	Soft
0.25 NAA + 2.0 Kin	50% (+); 50% (++)	Brown	Soft
0.5 NAA + 0.25 Kin	25% (+); 50% (++); 25% (+++)	Brown	Soft

0.5 NAA+0.5 Kin	12.5% (++); 37.5% (+++)	Brown	Soft
0.5 NAA+1.0 Kin	50% (+)	Brown	Soft
0.5 NAA+2.0 Kin	50% (+)	Brown	Soft
1.0 NAA+0.25 Kin	12.5% (+); 37.5 (++); 50% (+++)	Brown	Soft
1.0 NAA+0.5 Kin	75% (++);12.5% (+++)	Brown	Hard
1.0 NAA+1.0 Kin	50% (+); 25% (++); 25% (+)	Brown	Hard
1.0 NAA+2.0 Kin	50% (+)	Brown	Hard
2.0NAA+0.25 Kin	50% (+++); 50% (++)	Brown	Hard
2.0 NAA+0.5 Kin	37.5% (++); 50% (+++)	Brown	Hard
2.0 NAA+1.0 Kin	50% (+); 50% (++)	Brown	Hard

Table 5: Percentage of callus formation on MS media supplemented with 2, 4-D (0-2.0 mg/l) and BAP (0-2.0 mg/l), after six weeks of culture

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0.0 2,4-D	0%		
0.25 2,4-D	50% (+++); 50% (+++)	Grey	Soft
0.5 2,4-D	50% (++)	Grey	Soft
1.0 2,4-D	50% (+); 50% (++)	Grey	Soft
2.0 2,4-D	50% (+)	Grey	Soft
0 .0 BAP	0%		
0.25 BAP	25% (+)	Brown	Hard
0.5 BAP	25% (+)	Brown	Hard
1.0 BAP	25% (+)	Brown	Hard
2.0 BAP	25% (+)	Brown	Hard
0.25 2,4-D +0.25 BAP	100% (++++)	Grey	Soft
0.25 2,4-D + 0.5 BAP	25% (+++); 25% (+++)	Grey	Soft
0.25 2,4-D + 1.0 BAP	12.5% (++). 50% (+++); 37.5% (+++)	Grey	Soft
0.25 2,4-D+ 2.0 BAP	37.5% (++); 37.5% (+++)	Grey	Soft
0.5 2,4-D+ 0.25 BAP	75%% (+++)	Grey	Soft
0.5 2,4-D + 0.5 BAP	12.5% (+++); 87.5% (++++)	Grey	Soft
0.5 2,4-D + 1.0 BAP	25% (+++);25% (+++)	Grey	Soft
0.5 2,4-D+ 2.0BAP	12.5% (++); 25% (+++); 25% (++++)	Grey	Soft
1.0 2,4-D + 0.25 BAP	25% (+++);75% (++++)	Brown	Soft
1.0 2,4-D + 0.5 BAP	12.5% (+); 50% (++); 25% (+)	Brown	Soft
1.0 2,4-D+ 1.0 BAP	62.5% (+++); 25% (+)	Brown	Soft
1.0 2,4-D + 2.0 BAP	50% (++)	Brown	Soft
2.0 2,4-D+ 0.25 BAP	37.5% (++)	Brown	Soft
2.0 2,4-D+ 0.5 BAP	50% (+)	Brown	Soft
2.0 2,4-D+ 1.0 BAP	12.5% (++); 12.5% (+++); 12.5% (++)	Brown	Soft
2.0 2,4-D + 2.0 BAP	12.5% (+);25% (++)	Brown	Soft

Table 6: Percentage of callus formation on MS media supplemented with 2, 4-D (0-2.0 mg/l) and Kinetin (0-2.0 mg/l), after six weeks of culture

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0.0 2, 4-D	0%		
0.25 2, 4-D	50% (+++)	Grey	Soft
0.5 2, 4-D	50% (++)	Grey	Soft
1.0 2, 4-D	50% (+); 50% (+++)	Grey	Soft
2.0 2, 4-D	0%		
0.0 Kin	0%		
0.25 Kin	0%		
0.5 Kin	0%		
1.0 Kin	0%		
2.0 Kin	0%		
0.25 2, 4-D +0.25 Kin	50% (+++)	Grey	Soft
0.25 2, 4-D + 0.5 Kin	100% (++++)	Grey	Soft
0.25 2, 4-D + 1.0 Kin	25% (+); 50% (+++)	Grey	Soft
0.25 2, 4-D + 2.0 Kin	75% (++); 25% (+++)	Grey	Soft
0.5 2, 4-D+ 0.25 Kin	50% (+++)	Grey	Soft
0.5 2, 4-D + 0.5 Kin	50% (+++)	Grey	Soft
0.5 2, 4-D + 1.0 Kin	50% (+++)	Grey	Soft
0.5 2, 4-D + 2.0 Kin	50% (++)	Grey	Soft
1.0 2, 4-D+ 0.25 Kin	100% (+++)	Grey	Soft
1.0 2, 4-D + 0.5 Kin	12.5% (+);37.5% (+++);50% (+++)	Grey	Soft
1.0 2,4-D + 1.0 Kin	37.5% (++); 62.5% (+++)	Grey	Soft
1.0 2,4-D+ 2.0 Kin	50% (++); 50% (+++)	Grey	Soft
2.0 2,4-D + 0.25 Kin	50% (++); 50% (+++)	Grey	Soft
2.0 2,4-D+ 0.5 Kin	25% (++)	Grey	Soft
2.0 2,4-D + 1.0 Kin	25% (++)	Grey	Soft
2.0 2,4-D+ 2.0 Kin	25% (++)	Grey	Soft

Table 7: Percentage of callus formation on MS media supplemented with Picloram (0-2.0 mg/L) and BAP (0-2.0 mg/L), after six weeks of culture

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0.0 Pic	0%		
0.25 Pic	50% (+++);50% (+++)	Grey	Soft
0.5 Pic	100% (++++)	Grey	Soft
1.0 Pic	75% (+++)	Grey	Soft
2.0 Pic	75% (+++)	Grey	Soft
0.0 BAP	0%		
0.25 BAP	25% (+)	Brown	Hard
0.5 BAP	100% (+)	Brown	Hard
1.0 BAP	100% (+)	Brown	Hard
2.0 BAP	100 (+)	Brown	Hard
0.25 Pic+0.25 BAP	12.5% (+);75% (++);12.5% (+++)	Grey	Soft
0.25 Pic+0.5 BAP	87.5% (++); 12.5% (+++)	Grey	Soft
0.25 Pic+1.0 BAP	100% (++)	Grey	Soft
0.25 Pic+2.0 BAP	100% (++)	Grey	Soft
0.5 Pic+0.25 BAP	12.5% (+++); 87.5% (+++)	Brown	Hard
0.5 Pic+0.5 BAP	87.5% (+++);25% (++)	Brown	Hard
0.5 Pic+1.0 BAP	100% (+)	Brown	Hard
0.5 Pic+2.0BAP	100% (+)	Brown	Hard
1.0 Pic+0.25 BAP	50% (++); 50% (+++)	Brown	Hard
1.0 Pic+0.5 BAP	75% (++); 25% (+++)	Brown	Hard
1.0 Pic+1.0 BAP	75% (++); 25% (+++)	Brown	Hard
1.0 Pic+2.0 BAP	12.5% (++); 87.5% (+++)	Brown	Hard
2.0 Pic +0.25 BAP	100% (+++)	Brown	Hard
2.0 Pic+0.5 BAP	100% (+++)	Brown	Hard
2.0 Pic+1.0 BAP	25% (++): 25% (+++); 25% (++)	Brown	Hard
2.0 Pic+2.0 BAP	75% (+); 25% (++)	Brown	Hard

Table 8: Percentage of callus formation on MS media supplemented with Picloram (0-2.0 mg/L) and Kinetin (0-2.0 mg/L), after six weeks of culture

PGRs	Intensity of callus formation	Colour of callus	Texture of callus
0.0 Pic	0%		
0.25 Pic	50% (++)	Grey	Soft
0.5 Pic	100% (++++)	Grey	Soft
1.0 Pic	100% (++)	Grey	Soft
2.0 Pic	50% (+); 50% (++)	Grey	Soft
0.0 Kin	0%		
0.25 Kin	0%		
0.5 Kin	0%		
1.0 Kin	0%		
2.0 Kin	0%		
0.25 Pic+0.25 Kin	62.5% (+++); 37.5% (+++)	Grey	Soft
0.25 Pic+ 0.5 Kin	12.5% (+);50% (++); 37.5% (+++)	Grey	Soft
0.25 Pic + 1.0 Kin	50% (+0; 50% (+++)	Grey	Soft
0.25 Pic+ 2.0 Kin	50% (+); 50% (++)	Grey	Soft
0.5 Pic+ 0.25 Kin	50% (+); 50% (+++)	Brown	Soft
0.5 Pic + 0.5 Kin	50% (++); 50% (+++)	Brown	Soft
0.5 Pic+ 1.0 Kin	75% (++)	Brown	Soft
0.5 Pic + 2.0 Kin	50% (+); 50% (++)	Brown	Soft
1.0 Pic+ 0.25 Kin	75% (++)	Brown	Soft
1.0 Pic+ 0.5 Kin	75% (++)	Brown	Soft
1.0 2,4-D + 1.0 Kin	50% (++)	Brown	Soft
1.0 Pic+ 2.0 Kin	50% (++)	Brown	Soft
2.0 Pic+ 0.25 Kin	50% (++)	Brown	Soft
2.0 Pic+ 0.5 Kin	25 (++)	Brown	Soft
2.0 Pic+ 1.0 Kin	25% (++)	Brown	Soft
2.0 Pic + 2.0 Kin	50% (+); 50% (+++).	Brown	Soft

Effect of 2, 4-D with the presence of BAP or Kinetin in inducing callus: In Table 5, callus induced from leaf explants place on MS media supplemented with various concentration of 2, 4-D and BAP (0.0 mg/l – 2 mg/l) produced a mixture of greyish and soft callus or brown and hard callus depending on the concentration of the auxin and cytokinin used. In this experiment, 2, 4-D alone was able to induce callus (50%) and BAP alone also induced callus but at much lower growth, with 25% of callus proliferated on the leaf explants. MS+BAP alone gave rise to brown and hard callus. MS media supplemented with a lower concentration of 2, 4-D (0.25 mg/l - 0.5 mg/l) and BAP (0.25 mg/l - 2.0 mg/l) were able to initiate greyish and soft callus. As the concentration of 2, 4-D increased (1.0 mg/l to 2.0 mg/l) frequency of callus formation was lower and turned brown. The highest frequency of callus induction was obtained on MS+2, 4-D (0.25 mg/l) + BAP

(0.25 mg/l).

In Table 6, leaf explants placed on MS media added with various concentration of 2, 4-D (0mg/l-2.0 mg/l) and Kin (0mg/l - 2.0 mg/l) formed greyish and soft callus. In MS + Kin alone, no callus was observed in the leaf explants but the presence of 2, 4-D only, did induce callus formation. Low concentration of 2,4-D (0.25 mg/l - 0.5 mg/l) and low concentration of Kinetin (0.25 mg/l - 0.5 mgl) gave rise to more callus formation and the frequency of callusing reduced as the concentration of 2, 4-D (1.0 mg/l to 2.0 mg/l) and Kinetin (1.0 mg/l - 2.0 mg/l) increased. The highest frequency of callus was recorded in MS+ 2, 4-D (0.25 mg/l) + Kin (0.5 mg/l).

Effect of Picloram with the presence of BAP or Kinetin in inducing callus: In the experiment where Picloram + Kin or BAP were added to MS media, Pic (0.5 mg/l) was adequate to induce high frequency of callus on leaf explants (Table 7 and Table 8). All leaf explants placed on MS media containing 0.25, 0.5, 1.0 and 2.0 mg/l Pic alone were able to induce callus which were soft and greyish. Low concentration (0.25mg/l) of Pic combined with BAP (from 0.25 mg/l - 2.0mg/l) induced callus which were grey and soft (Table 7). However, higher concentration of Pic (0.5 mg/l) and BAP (0.25 mg/l - 2.0 mg/l) formed calli that were brown and hard.

A mixture of grey and brown callus was induced when leaf cuttings were placed on varying combination of Pic and Kin (Table 8). Similar to Pic-BAP, lower concentration of Pic (0.25 mg/l) combined with Kin (0.25 mg - 2.0 mg/l) induced callus which were grey and soft. However, increase in Pic concentration (1.0 mg/l to 2.0 mg/l) along with Kin, only lower frequency of callusing was recorded, and the callus produced were brownish in color. Highest percentage callus was observed on MS +Pic (0.5 mg/l).

Best media for callus proliferation

Based on the above six combination of MS + auxin-cytokinin treatment for callus induction, media combination that gave the highest percentage of callus were selected and further tested for highest biomass induction. These selected best media were evaluated for the increase in callus biomass, reading taken every 8 weeks during subculture. The increase in biomass were calculated based on difference in fresh weight of callus obtained at the end of week-16 (2^{nd} subculture) and week-24 (3^{rd} subculture) (Table 9). Media devoid of supplemented cytokinin, MS + Pic (0.5mg/l) gave the highest percentage of increase in callus proliferation, 248.04%. This was followed by MS+2,4-D (0.25mg/l) +Kin (0.5mg/l) with an increase of 101.20%. The MS media supplemented with NAA/BAP and NAA/Kin gave the lowest increase in callus biomass upon three subcultures with 27.27% and 21.27% respectively. Media, MS+2,4-D (0.25mg/l) + Kin (0.5mg/l) showed a better outcome increase (101.20%) compared to the increase in callus weight from callus obtained from MS +2,4-D (0.5m/l) + BAP(0.25mg/l) which gave an increase of 50.86%.

Table 9: Percentage of increase in callus biomass (%) of *C. nutans* leaf explant, on selected MS media + plant growth regulators from each treatment block (n=4)

PGR	Concentration of PGRs (mg/L)	Callus texture and color	FW1 (mean± sd)/g	FW2 (mean± sd)/g	FW3 (mean± sd)/g	Increase in biomass (%) (FW3-FW2) /FW2
NAA+BAP	2.0:0.00	Brown/hard	0.13 ± 0.03^{b}	0.22 ± 0.04d	0.28 ± 0.04^{d}	27.27
2,4-D+BAP	0.5:0.25	Grey/friable	0.16 ± 0.03^{b}	1.16 ± 0.23^{a}	1.751 ± 0.03^{a}	50.80
PIC+BAP/KIN	0.5:0.00	Grey/friable	0.17 ± 0.03^{b}	0.51 ± 0.06^{c}	1.775 ± 0.07^{a}	248.04
NAA+KIN	0.25:1.0	Brown/friable	0.14 ± 0.03^{b}	0.94 ± 0.29^{b}	1.14 ± 0.25^{c}	21.27
2,4-D+KIN	0.25:0.50	Grey/friable	0.32 ± 0.03^{a}	0.83 ± 0.09^{b}	1.67 ± 0.11^{b}	101.20

*Means of different values shown by different letters in the same column are statistically different using Duncan's multiple range test at 0.05 level of significance

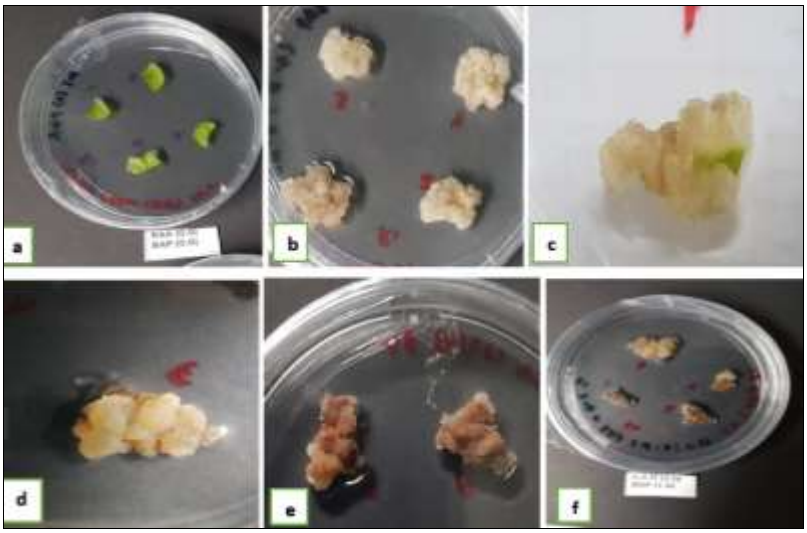

Fig 2: Various callus induced in different PGRs used;(a)Leaf cuttings placed on MS media without any PGRs; (b) Callus induced on explants in MS+2, 4-D (0.5 mg/l)+BAP (0.25 mg/l); (c) Callus induced on explants in MS+ Pic (0.5 mg); (d) Callus formed on explants in MS+ 2, 4-D (1.0 mg/l) + Kin (0.25 mg/l); (e) Hard brown callus formed on explants in MS+NAA (2.0 mg/l) alone; (f) Hard callus formed on MS+ 2, 4-D (2.0 mg/l) +BAP (1.0 mg/l).

Discussion

As a preliminary step, sterile and clean plant materials for *in vitro* induction work is crucial to maintain contamination free cultures. This is to provide a homogenous plant material which is not dependent on the environmental constraints besides establishing microbe free Clutures (Khosravi *et al.*

2007) [39]. Successful *in vitro* plantlets were established using triple surface sterilization steps that involved 70% ethanol and double Clorox® treatment. Natrium hypochlorite (NaOCl) has a wide range of anti-microbial activity, and it is potent against spores, fungi and bacteria (Lazo-Javalera *et al*. 2016) [40]. The use of Clorox is popular compared to natrium hypochlorite powder dissolved in water as this home bleach contains 5.2% natrium hypochlorite (NaOCL) and hypochlorous acid (HOCl) that helps in the plant surface disinfection process (Waheeda and Shyam, 2017) [41]. Besides, hypochlorous acid (HOCL) also denatures the essential protein in the microbes that could possibly contaminate the plant (Ramalashmi *et al.* 2018) [42]. Prolonged exposure time (50 minutes) at a lower concentration of Clorox® allows the penetration of the sterilant agent throughout the different layers of meristematic tissues of the exposed nodal cuttings. Since *C. nutans* has soft stem tissues, a prolonged exposure at a higher concentration of Clorox causes a decrease in viable clean culture indicating tissue damage or browning of tissues. Similar plant sterilization approach, double sterilization step using Clorox was used in unpublished work by Gunasekaran (2014) [43] to obtain high rate of viable clean cultures. In this study, more than 70% viable nodal cuttings were obtained using double treatment of Clorox® ranging 30 to 50% concentration. This contradicts with the findings from Hashim *et al* (2021) [33] outcome where low viability of *C. nutans* just with a single application of Clorox® ranging 10% to 40% was reported. However, in an unpublished work by Rou *et al* (2015) [44] reports 100% success using single application of low concentration of Clorox for *C. nutans*. The variation in the sterilization techniques as in using single sterilization, or double or even triple is subjective to the source and type of the explants (Tiwari, 2016) [45]. Moreover, matured plants can withstand harsh application of sterilizing agents compared to juvenile plants (Waheeda and Shyam, 2017) [41]. The mother plant used in this study was eight years old field grown plants. Therefore, it may be more prone to carry microbes and thus requires higher concentration of disinfectant. It must also be able to withstand the effect of higher concentration of sterilant owing to matured meristem. Successful sterilizing method using triple step was reported in other medicinal plants such as in *Calligonum polygonoides* (Owis *et al*., 2016) [46].

The effect of plant growth regulators on the callusing effect of *C. nutans* had exhibited a variety of callus formation. Fresh weight of callus obtained proves the cell division that occurs in the cut leaves during callugenic effect and the higher the weight of callus shows more callus augmentation (Suhartanto *et al*. 2022) [47]. Highest increase in callus biomass was with MS added with Picloram only. This less explored herbicide which owns growth regulating characteristics (Collins *et al.* 1978) [48], alone or in combination with various cytokinins (Kinetin or BAP) was able to induce callus on *in vitro* derived *C. nutans* leaf explants. Hence, the callus induced which was friable and greyish could be used for cell suspension establishment in future as the friable callus could easily disperse and multiply in cell suspension. Friable callus shows that it has a high-water content in its cells. Picloram plays a vital role in cell elongation, helps in loosening the cell wall and detach the wall bonding of a cell which makes it easier for the formation of callus (Eisinger and Morre, 1971) [49]. This agrees with the findings by Genady (2017) [50] where Picloram individually outperformed 2, 4-D to induce callus in *Verbena bipinnatifida*. Similar findings of Picloram being able to induce high frequency of callus was reported in *Simmondsia chinensis* (Bekheet *et al*., 2018) [51] and *Cheilocostus speciosus* (Hundare *et al*., 2017) [52].

In the MS media supplemented with 2,4-D along with Kinetin or BAP, callus was also successfully induced. This is in line with Bong *et al* (2021) [34] where highest callus induction was obtained from *C. nutans* leaves placed on MS media added with 2, 4-D (0.5mg/l) and BAP (0.25mg/l). It has been reported that 2, 4-D, at lower concentration reacts as in other exogenous hormones and induces callus, and at higher concentration it functions as herbicide (Davis, 1997) [53] thus slows down the rate of callusing in plants. The callus obtained were also brown in colour indicating high phenolic compounds being presence in this plant. Study conducted by Phua *et al*. (2016) [34], reported that leaf explants of *C. nutans* formed callus on MS media supplemented with 2,4-D alone (0.5mg/l). This finding contradicts the present study. This could be because the source of explant being different as in this study, *in vitro* grown *C. nutans* leaf explants were used and the study conducted by Phua *et al* (2016) [34], fresh field grown leaf explants were surface sterilized. Tissue cultured leaf explants respond better to plant growth regulators in media compared to the field grown leaf explants due to the difference in the pre-available endogenous growth regulators (Krishnan *et al*., 2018) [54]. Other studies testing PGR combination (2, 4-D and Kinetin) reported to be effective in producing callus are in medicinal plants, such as *Aquilaria malaccensis* (Saikia *et al.*, 2012) [55] and *Barrington racemose* (Osman *et al*. 2016) [56].

The presence of cytokinin alone was not appropriate to induce callus in *C. nutans*. Limited callus induction using Kinetin or BAP have been reported, for example Kinetin did not induce callus formation in *Barringtonia racemosa* leaf and endosperm, but BAP produced brown and hard callus (Dalila *et al*., 2013) [57]. However, *Orthosiphon stamineus* did not induce any callus when placed on BAP alone supplemented media (Elangomathavan *et al*., 2017) [58]. This clearly indicates, both cytokinins, Kinetin and BAP need to be in synergistic action with an auxin such as 2, 4-D or NAA to induce callus at some rate (Ikkeuchi *et al*., 2013) [29].

In the current study, combination of NAA and BAP was not effective to induce callus with compact and brownish callus formed. The browning effect of the callus could be due to the oxidation effect from the phenolic compound in *C. nutans*. The build-up of phenolic compound is produced as the plant's defense mechanism against microbial infection or physiological stress which is formed due to the wounding of the leaf explant (Ling *et al.*, 2009) [59]. NAA can initiate stress in wounded plant tissues, thus inhibiting proper callus proliferation (Aslam *et al*., 2015) [60]. Similarly, the presence of NAA alone did not induce callus in *Matthiola incana* (Kaviani *et al*., 2013) [61]. Likewise, NAA and BAP had failed to induce callus in other plants such as *Haworthia* (Kaul and Sabharwal, 2002) [62] but was effective in *Atropa acuminata* (Dar *et al*., 2021) [63] and *Lavatera cashmeriana* (Wani *et al*., 2018) [64]. However, with the presence of Kinetin, in MS media added with NAA did exhibit higher callus formation. Similar findings had been reported by Sayadi *et al.* (2014) [65] where combined effect of NAA and Kinetin induced callus from *Matricaria chamomilla*.

Conclusion

Mass propagation of *C. nutans* can be carried out using the protocol tested here which could provide seasonal free and disease-free plantlets. The induced friable callus obtained in this study using Picloram as growth hormone has the potential

to be furthered as cell suspension and cultivation in bioreactors. This could help minimize the exploitation of the wild grown *C. nutans* that could lead to extinction.

Statement and declaration

Competing interest

The authors have not declared any conflict of interests

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors to conduct this study.

Acknowledgement

The authors appreciate and thank School of Bioscience, Faculty of Health and Medical Sciences, Taylor's University, Malaysia for the technical support and facilities provided.

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