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Effects of growth regulators on production of bioactive compounds from *Clinacanthus nutans* (Burm.f.) Lindau "Phaya Yo" culture *in vitro*

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ABSTRACT: *Clinacanthus nutans* is a valuable plant that is harvested for medicinal purposes in Southeast Asia. However, the production of herbal medicines needs raw, uncontaminated materials with a consistent quantity of bioactive compounds. Therefore, *in vitro* culture is of interest for the production of secondary metabolites. The present study applied plant growth regulators to stimulate bioactive compounds from multiple shoots of *Clinacanthus nutans*. Nodal explants were studied for multiple shoot induction using different types of plant growth regulators: thidiazuron (TDZ) alone or in combination with 1-naphthaleneacetic acid (NAA). The obtained calli or multiple shoots were examined for bioactive compounds (total phenolic, flavonols/flavones, phytosterols and antioxidant activity). The results showed that TDZ at concentrations of 0.5–1.0 mg/l either alone or in combination with NAA at concentrations of 0.01–0.1 mg/l stimulated a higher total phenolic content (including antioxidant activity) than the control. At such concentrations, TDZ and NAA were more likely to stimulate lupeol production in the tissue and its content was higher than in the leaves from the pot-grown plants. The present result revealed suitable growth regulator factors for stimulating the production of bioactive compounds, such as total phenolic compounds, antioxidants and phytosterols and especially lupeol, which were found in the tissues of *C. nutans* at a level 98.25-folds higher than in the leaves from the pot-grown plants. This technique could support the production of raw materials containing known quantities of various bioactive compounds at the laboratory level and be used for further research on the creation of quantitative bioactive compounds in *C. nutans* herbs for commercial production.

KEYWORDS: *Clinacanthus nutans*, medicinal plants, plant growth regulator, 1-naphthaleneacetic acid, thidiazuron, bioactive compounds

INTRODUCTION

Clinacanthus nutans (Burm.f.) Lindau "Phaya Yo" is commonly known as Sabah snake grass which is one of the most important medicinal plants belonging to the family Acanthaceae. It is native to many tropical Asian countries, including Malaysia, Thailand, Indonesia and China [1]. It is used as a traditional medicine in the treatment of skin diseases to treat insect bites, anti-inflammatory, anti-allergy and antiviral activity against herpes simplex virus type-2 and varicella zoster virus [2, 3]. To date, this plant has received much attention from many researchers for its medicinal potency. The Thai Ministry of Public Health considered this plant as a main remedy for the treatment of skin inflammations and lesion caused by viruses [4]. The compounds showing this anti-viral activity have been reported as glycoglycerolipids, such as monogalactosyl diglycerides and digalactosyl diglyceride [5, 6]. The other active substances from *C. nutans* leaves are stigmasterol, lupeol, β-sitosterol, betulin, 6 types of C-glycosyl flavones (vitexin, isovitexin, shaftoside, isomollupentin-7-O-β glucopyranoside, orientin and isoorientin) and sulfur-containing glucosides [7–10]. The phytosterols, such as stigmasterol, lupeol and β-sitosterol, have shown anti-cancer, anti-inflammatory and antimicrobial activity [11, 12]. Nowadays, the large group of compounds of interest is the phenolics and flavonoids with antioxidant activity. This group of compounds can be applied as an immune booster and in skin care products. As a result, there is an increasing demand for *C. nutans* leaves to make various medicinal products; however, the problems of conventional cropping are poor quality raw materials, such as fungal or chemical contamination, and inconsistent bioactive ingredients in each crop. Plant tissue culture is an alternative for the production of secondary metabolites under controlled conditions [13, 14]. For *C. nutans*, there are some reports of shoot induction using 6-benzylaminopurine (BAP) and 1-naphthaleneacetic acid (NAA) which produced 1.67 and 3.9 shoots, respectively [15, 16]. However, studies on other species, which belong to the same family, mentioned that using thidiazuron (TDZ) instead of BAP in combination with NAA was able to produce a higher number of shoots [17, 18]. In addition, TDZ acts as an effective elicitor in the production of secondary metabolites in plant tissue culture [19].

The present study aimed to develop a method for culturing *C. nutans* tissue to create bioactive compounds or secondary metabolites by using two plant growth regulators (auxin: NAA and cytokinin: TDZ) and to analyze the bioactive compounds (total phenolic content, flavonols/flavones content, antioxidant activity and phytosterol) in multiple shoot clusters and leaves from pot-grown plants using spectrophotometry and high-performance liquid chromatography (HPLC). The output should be useful in the production of quantitatively important substances leading to future industrial production.

MATERIALS AND METHODS

Plant materials and sterilization

Branches aged 3–4 months with 2–3 nodes from *C. nutans* were used as explants. The explants were washed, disinfected using 10% and 5% Clorox solution (5.25% sodium hypochlorite) added with 1–2 drops of Tween 20 for 10 min and 5 min, respectively, and rinsed twice with sterile distilled water. Then, the aseptic explants were excised into pieces about 1 cm long with 1 node each and cultured in Murashige and Skoog (MS) medium [20], without growth regulators but supplemented with 30 g/l sucrose [16] and 8.0 g/l agar with the pH adjusted to 5.6–5.8. The nodes were placed under 37 µmol/m^2/s light intensity for 8 h/day in a room where the temperature was controlled at 28 °C for 7 days.

Shoot and callus induction

The explants of *C. nutans* were cultured on MS medium supplemented with growth regulators: TDZ at concentrations of 0, 0.5 or 1.0 mg/l in combination with NAA at concentrations of 0, 0.01 or 0.1 mg/l alone or in combination. Both the TDZ and NAA used were of analytical grade (Sigma-Aldrich Co.; St Louis, MO, USA). The MS basal medium without growth regulators served as the control. All the experiments were conducted with three replications and eight explants/replication. The cultures were maintained under the same culture conditions mentioned above. The data on the shoot regeneration percentage, number of shoots and callus induction were recorded after incubation for 2 months. The mean difference in each trial was compared based on Duncan's multiple range test, with significance tested at the $p \leqslant 0.05$ level.

Shoot and initiated callus were subcultured to the same media using continuous culture for 2 months to increase the number of multiple shoot clusters. Each multiple shoot cluster was used to examine the total phenolic content, flavonols/flavones content and antioxidant activity using spectrophotometry and the phytosterol content using high-performance liquid chromatography (HPLC).

Sample preparation for quantification of bioactive compounds

Multiple shoot clusters (aged 3–4-months) from the tissue culture and the fully expanded leaves at node 3–4 from the pot-grown plants (watered every second day and fertilized every month under full sunlight) were collected and dried in an oven at 60 °C for 24 h or until the weight remained constant. The dried samples were ground into powder (<250 µm). The extraction was modified from the Akbel and Bulduk procedure [21]. Each sample (0.1 g) was extracted with 5 ml of 80% methanol, vortex mixed for 10 s and then placed in a sonicator for 15 min. The mixtures were left in the refrigerator for 15 h and then centrifuged at 11,000 rpm and 4 °C for 10 min. The supernatant was used for determination of the total phenolic, flavonols/flavones and antioxidant capacity.

Determination of total phenolic content

The total phenolic content was determined based on the Folin-Ciocalteu method modified from Mohd-Esa et al [22], using gallic acid as a standard. A sample (20 µl) of the extract was pipetted into a 96-well plate; then, 100 µl of 10% Folin-Ciocalteu reagent was added to each well, followed by 80 µl of 7.5% Na_2CO_3. The mixture was left for 30 min and the absorbance was measured at a wavelength of 765 nm using a microplate reader. The phenolic content was reported as milligrams equivalent to gallic acid per 1 g dry weight (mg GAE/g DW).

Determination of flavonols and flavones content

The flavonols/flavones content was determined using the aluminum chloride ($AlCl_3$) method modified from Pourmorad et al [23], with quercetin as a standard. A sample (1 µl) of extract was pipetted into a 96-well plate and each well was added with 5 µl of 10% $AlCl_3$ and 5 µl of 1 M potassium acetate, before adding 140 µl of 80% methanol and mixing well. The mixture was left at room temperature for 30 min after which the absorbance was measured at a wavelength of 415 nm using a microplate reader. The flavonols/flavones content was reported as milligrams equivalent to quercetin per 1 g dry weight (mg QE/g DW).

Determination of antioxidant activity

Antioxidant activity analysis was measured using the diphenyl-picrylhydrazyl (DPPH) radical scavenging method, according to Sittisart et al [24]. A sample (100 µl) of extract or standard reagent (trolox) was pipetted into a 96-well plate and each well was added with 100 µl of 0.2 mM DPPH solution and left for 30 min in the dark. The absorbance at a wavelength of 517 nm was measured using a microplate reader. The antioxidant activity was calculated based on the percentage of the disappearance of the purple color. The percentage of DPPH inhibition was calculated from

the equation % DPPH = $(A_{blank} - A_{sample})/A_{blank} \times 100$, where A_{blank} is the absorbance of the solvent (80% methanol) after reaction with DPPH and A_{sample} is the absorbance of the sample or standard after reaction. The DPPH inhibition percentages of extracts were compared to the standard curve and antioxidant activity was reported as milligrams equivalent to trolox per 1 g dry weight (mg TE/g DW).

Analysis of phytosterols using HPLC

Extraction of phytosterols using methanol was modified from the Jaber and Jasim method [25]. A dry powder sample (0.2 g) was macerated with 10 ml methanol in a 50 ml tube with a screw cap. The suspension was mixed for 1 min using a vortex mixer and then placed in an ultrasonic bath for two periods of 30 min. The macerated samples were kept in a refrigerator for 12 h. This suspension was filtered through Whatman No. 1 filter paper using a suction pump. The methanolic filtrate was evaporated using a rotary evaporator until it was dry. This crude extract was dissolved in methanol (HPLC grade) and the volume was adjusted to 1 ml. The extract was passed through a nylon membrane filter (0.2 µm, 13 mm diameter) before injection for HPLC. The HPLC system consisted of a controller (Waters 600), pump (600 E), autosampler (717 plus) and photodiode array detector (2998), all from Waters (Waters Corporation; Milford, MA, USA). An isocratic elution was performed on a Luna® 5 µm C18(2) 100A, 250 × 4.6 mm (Serial # H19-266426; Phenomenex; Torrance, CA, USA) with a guard cartridge (C18; 4 × 3.0 mm internal diameter). The mobile phase was a mixture of acetonitrile and ethanol (40:60, v/v). This method was modified from the guidelines of Shailajan et al [26]. Injection volumes were 20 µl and the flow rate was 1 ml/min. The UV detector wavelength was set at 210 nm for the determination of lupeol, stigmasterol and β-sitosterol. Quantification was achieved by comparison of the retention time and the peak-area ratio with external standards of lupeol, stigmasterol and β-sitosterol (> 95% pure; Sigma-Aldrich Co.) at concentrations of 0–200 ppm.

RESULTS AND DISCUSSION

in vitro single node culture of *C. nutans* as affected by TDZ and NAA

The shoot inductions of nodal segments of *C. nutans* were cultured onto MS basal medium with TDZ at 0, 0.5 or 1.0 mg/l in combination with NAA at 0, 0.01 or 0.1 mg/l. All treatments were successful in producing 100% shoot regeneration and only the treatment with the TDZ supplement produced a cluster of small shoots. The MS basal medium (control) could induce shoots emerging from the lateral bud with a green color and produced a low number of shoots (1 shoot) with a shoot height of 4.22 cm. The treatment with 0.01 and 0.1 mg/l NAA alone produced 1.27 and 1.40 shoots, respectively, and shoot heights of 4.72 and 4.79 cm, respectively, which were not significantly different from the control (Fig. 1, Table 1). These results indicated that using NAA alone could not induce multiple shoots because NAA is a synthetic auxin that mainly controls cell elongation and root development [27].

Multiple shoots were observed in the MS media containing 0.5–1.0 mg/l TDZ alone or in combination with 0.01–0.1 mg/l NAA. The highest number of shoots (6.33) was produced using the treatment with 1.0 mg/l TDZ and 0.1 mg/l NAA, with a shoot height of 1.11 cm, which were both significantly different from the control and MS with NAA alone (Fig. 1, Table 1). In the single node culture of *C. nutans*, shoot induction was affected by the application of cytokinin in the culture medium. This result was consistent with another report in which multiple shoots were induced by the combination of cytokinin (kinetin) and auxin (NAA) but not by auxin (NAA) alone [28]. In addition, similar results were reported by Chen et al [15], where a full-strength MS basal medium supplemented with 1.0 mg/l BAP and 0.02 mg/l NAA produced the highest average number (3.9) of shoots for *C. nutans*. Wang et al [29] found that the most suitable formula for culturing *C. nutans* tissue was MS medium supplemented with 1.0 mg/l BAP and 0.1 mg/l NAA. Haida et al [16] stated that MS medium supplemented with 12 µm BAP (2.7 mg/l) and 30 g/l sucrose provided the best option to induce the highest average number (1.67) of shoots and average height (3.23 cm). Based to these above-mentioned reports of *C. nutans* tissue culture, BAP alone or in combination with NAA was suitable to stimulate shoot multiplication. In the present study, TDZ was used as a cytokinin instead of BAP, which produced the highest average number (6.33) of shoots using 1 mg/l TDZ combined with 0.1 mg/l NAA. It is possible that TDZ is the most potent cytokinin for the induction of callus, multiple shoots and somatic embryos [30].

In addition, yellowish-green calli were formed at the base of nodes and there was regeneration at the shoot apex in the media with 0.5–1.0 mg/l TDZ either alone or in combination with 0.01–0.1 mg/l NAA (Fig. 2). Hence, the ratio of exogenous TDZ and NAA added in the basal medium affected shoot initiation derived from callus. It is known that a cytokinin applied exogenously in a basal medium can promote cell division and cell differentiation, while auxin is used to promote the cell elongation of explants [27]. However, TDZ has exhibited both auxin-like and cytokinin-like effects that are necessary for cell division and shoot regeneration [31]. A similar study was reported by Najar et al [32] for a nodal culture of the Indian herb *Tylophora indica*. They found that 1.5 mg/l of TDZ in combination with 0.1 mg/l of IAA could induce a

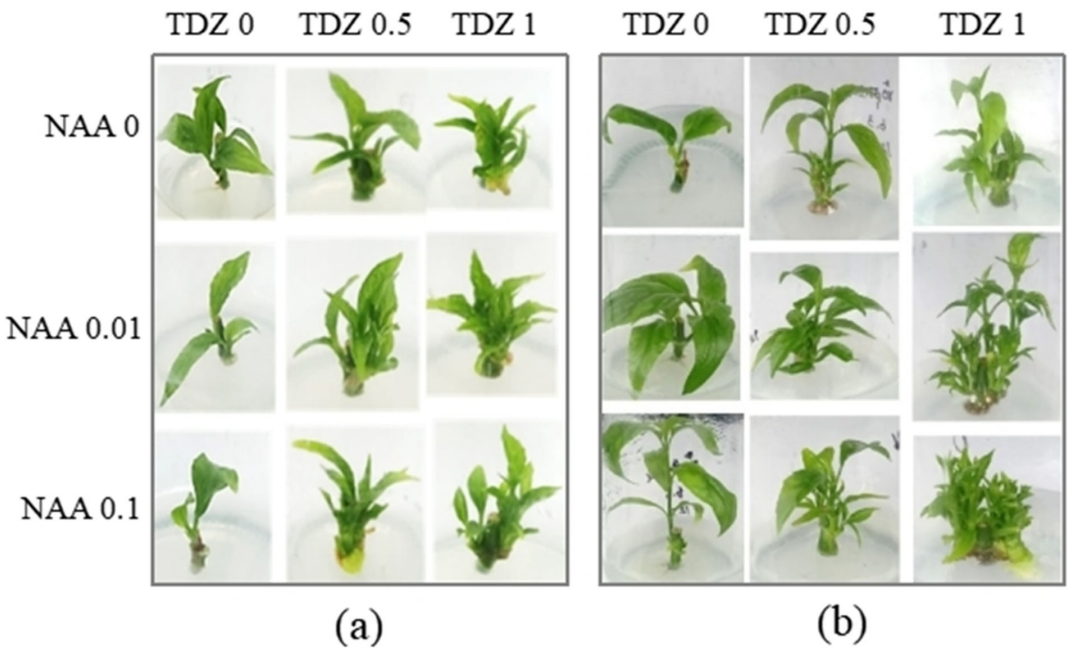

Fig. 1 Multiple shoots initiation from node cultures of *C. nutans* cultured on MS medium containing various concentrations (0.5 or 1.0 mg/l TDZ alone and in combination with 0.01 or 0.1 mg/l NAA) for (a) 1 month and (b) 2 months.

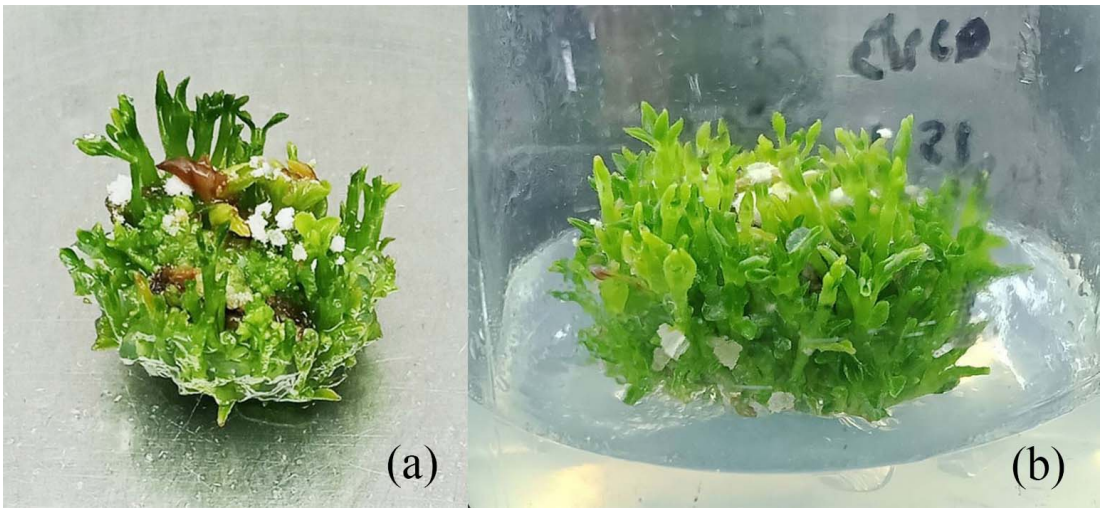

Fig. 2 Calli development of *C. nutans*; (a) apex regeneration into numerous shoots, (b) cultured onto MS medium with 0.5 mg/l TDZ combined with 0.1 mg/l NAA.

number of shoots, with a good shoot length and callus formation at the base of the explant. At higher IAA concentrations, the callus size was enlarged. Addition of TDZ to the medium was more effective in tissue culture than other cytokinins. Prathanturarug et al [33] reported that the use of TDZ had an effect on inducing multiple shoots in *Curcuma longa* cultures. Auxin stimulates cell elongation and rooting, including an endogenous auxin promoting callus formation [34]. Similarly, there have been reports of callus induction and shoot development in many medicinal plants. Licorice (*Glycyrrhiza* spp.) tissue culture, using TDZ at low concentrations of 0.1–0.5 mg/l could induce callus formation and develop shoots [35]. *in vitro* culture of the leaf and stem tissue of qinghao (*Artemisia annua*) in MS medium supplemented with 0.1 mg/l TDZ alone stimulated the production of new shoots, with the highest number being 57 shoots/explant [36].

Table 1 Effect of various concentrations of TDZ and NAA on shoot regeneration percentage, number of shoots, height of shoots and number of nodes inducing callus of *C. nutans* on MS medium for 2 months.

Growth regulator (mg/l)	Nodes for shoot regeneration (%)	Average shoot initiation/node[1]	Average height of shoot[1](cm)	Number of nodes inducing callus (%)
0 TDZ 0 NAA	100	1.00 ± 0.00^{c}	4.22 ± 2.28^{a}	0
0 TDZ 0.01 NAA	100	1.27 ± 0.46^{c}	4.72 ± 1.41^{a}	0
0 TDZ 0.1 NAA	100	1.40 ± 0.53^{c}	4.79 ± 2.30^{a}	0
0.5 TDZ 0 NAA	100	4.00 ± 1.73^{b}	1.00 ± 0.13^{b}	83.33
0.5 TDZ 0.01 NAA	100	5.33 ± 0.76^{ab}	1.17 ± 0.41^{b}	83.33
0.5 TDZ 0.1 NAA	100	5.33 ± 1.61^{ab}	1.27 ± 0.62^{b}	91.67
1 TDZ 0 NAA	100	4.33 ± 0.34^{b}	1.00 ± 0.18^{b}	83.33
1 TDZ 0.01 NAA	100	5.67 ± 0.76^{ab}	1.63 ± 0.32^{b}	91.67
1 TDZ 0.1 NAA	100	6.33 ± 0.29^{a}	1.11 ± 0.35^{b}	100

[1]Means ± standard deviation of three replicates ($n = 3$) within a column followed by the same lowercase superscript are not significantly different based on Duncan's multiple range test ($p \leqslant 0.05$).

Total phenolic, flavonols and flavones content and antioxidant activity from multiple shoot clusters of *C. nutans*

The clusters of multiple shoots of *C. nutans* in each treatment were analyzed for the total phenolic content, flavonols/flavones content and antioxidant activities using a spectrophotometric technique. The total phenolic content was in the range 5.98–13.65 mg GAE/g DW. The low total phenolic group (5.98–7.25 mg GAE/g DW) was recorded from the MS medium (no growth regulator) and the treatment of NAA without TDZ. The high total phenolic group (11.98–13.65 mg GAE/g DW) was produced in all treatments with the TDZ supplement. The antioxidant activities in each treatment were measured using the DPPH method and were in the range 6.74–28.30 mg TE/g DW. In addition, high levels of DPPH scavenging activity (24.92–28.30 mg TE/g DW) were measured in all treatments with the TDZ supplement. Low levels of DPPH scavenging activity (6.74–11.64 mg TE/g DW) were measured in the leaves from the pot-grown plants and in all treatments without TDZ. The flavonols/flavones content of the *C. nutans* leaves from the pot-grown plants was highest compared to all the treatments derived from multiple shoots culture. However, the total phenolic content and antioxidant activity from multiple shoot clusters grown in MS medium with TDZ concentrations in the range 0.5–1.0 mg/l alone or in combination with NAA in the range 0.01–0.1 mg/l were significantly higher than in the leaves from the pot-grown plants (Table 2).

The growth regulator is the most important factor in shoot induction and calli formation in a single node culture of *C. nutans*. The use of growth regulators, such as TDZ at a concentration of 0.5–1.0 mg/l alone or in combination with 0.01–0.1 mg/l NAA, stimulated multiple shoots from the node and shoot regeneration from callus and increased the production of the total phenolic content and antioxidant activity to a higher level than for the leaves from the pot-grown plants. However, the leaves from the pot-grown plants contained more flavonols/flavones substances than the tissue culture. This result may have been associated with the mechanism mentioned in the study by Amoo and Staden [37] describing the supplementation of culture medium with cytokinin and low NAA concentrations that resulted in a significantly reduced flavonoid content, as a result of a possible antagonistic interaction of low NAA concentrations with cytokinin on flavonoid production *in vitro*. The antioxidant potential in several medicinal plants is essentially a result of phenolic compounds [38]. This correlated with the present results where the total phenolic content and antioxidant activity had the same trend. Similarly, Haida et al [16] revealed that the leaves of *C. nutans* from tissue cultures had total phenolic, flavonoid, alkaloids and tannin contents higher than those of conventionally propagated leaves. The concentration of secondary metabolites in plants may be stimulated by plant growth regulators. The present of TDZ alone or in combination with NAA induced the production of phenolics and antioxidant activity in multiple shoot clusters that was 2-folds higher than in the leaves from the pot-grown plants. This could have been due to the effect of a plant growth regulator, such as TDZ, which can stimulate stress response, with the early oxidative stress stimulating cell division, differentiation and morphogenesis [31]. In general, secondary metabolites have been shown to occur in response to various types of stress [39, 46]. Therefore, the accumulation of phenolic compounds in the *in vitro* culture might be associated with the stress response induced by the tissue culture conditions in the multiple shoot development process [40].

Phytosterol content in multiple shoots of *C. nutans*

The multiple shoot clusters of *C. nutans* in each treatment were analyzed for three phytosterol contents: lupeol, stigmasterol and β-sitosterol (Fig. 3). The MS medium with 0.5–1.0 mg/l TDZ, either alone or in

Table 2 Total phenolic and flavonols/flavone content and antioxidant activity in leaves from pot-grown plants and multiple shoot clusters of *C. nutans* cultured in media supplemented with growth regulators for 4 months.

Growth regulator (mg/l)	Bioactive substances in multiple shoot clusters[1]		
	TPC[2] (mg GAE/g DW)	TFF[3] (mg QE/g DW)	AA[4] (mg TE/g DW)
Leaves from pot-grown plants	6.14 ± 0.03^{b}	15.00 ± 0.62^{a}	6.74 ± 0.75^{c}
0 TDZ 0 NAA	5.98 ± 0.65^{b}	3.42 ± 0.21^{b}	8.37 ± 2.65^{bc}
0 TDZ 0.01 NAA	6.47 ± 0.30^{b}	3.68 ± 0.12^{b}	9.67 ± 0.48^{bc}
0 TDZ 0.1 NAA	7.25 ± 0.37^{b}	3.12 ± 0.62^{bc}	11.64 ± 1.48^{b}
0.5 TDZ 0 NAA	12.83 ± 2.57^{a}	2.34 ± 0.63^{de}	24.92 ± 4.90^{a}
0.5 TDZ 0.01 NAA	13.65 ± 1.15^{a}	2.13 ± 0.40^{de}	28.30 ± 1.03^{a}
0.5 TDZ 0.1 NAA	13.50 ± 1.06^{a}	2.16 ± 0.27^{de}	27.32 ± 1.87^{a}
1 TDZ 0 NAA	12.93 ± 1.37^{a}	2.00 ± 0.02^{e}	26.72 ± 2.32^{a}
1 TDZ 0.01 NAA	11.98 ± 3.26^{a}	2.33 ± 0.26^{de}	27.43 ± 2.19^{a}
1 TDZ 0.1 NAA	12.93 ± 0.77^{a}	2.63 ± 0.16^{cd}	26.00 ± 1.22^{a}

[1] Means ± standard deviation of three replicates ($n = 3$) within each column followed by the same lowercase superscript are not significantly different according to Duncan's multiple range test ($p \leqslant 0.05$). [2] TPC-Total phenolic compounds expressed in milligrams of gallic acid equivalents per gram of dry weight. [3] TFF-Total flavonols and flavones expressed in milligrams of quercetin equivalents per gram of dry weight. [4] AA-Antioxidant activity expressed in milligrams of trolox equivalents per gram of dry weight.

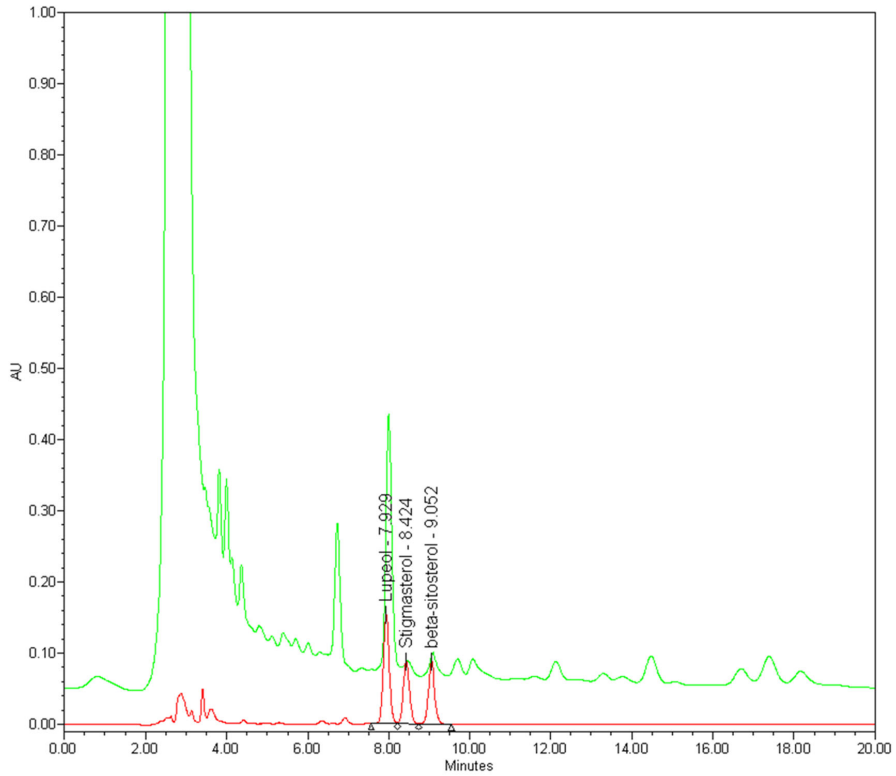

Fig. 3 Chromatogram of lupeol, stigmasterol and β-sitosterol standard (red line) compared to extract from multiple shoot clusters of *C. nutans* (green line).

combination with 0.01–0.1 mg/l NAA, tended to stimulate the production of lupeol (0.95–3.93 mg/g DW), while lupeol in the leaves from the pot-grown plants produced 0.04 mg/g DW. Notably, the lupeol induction from tissues was higher than from the leaves of the pot-grown plants by 23.75–98.25 times, with the MS medium with 0.5 mg/l TDZ and 0.1 mg/l NAA being the most effective conditions for the highest lupeol stimulation (3.93 mg/g DW) in the multiple shoot tissues of *C. nutans*. Stigmasterol and β-sitosterol were

Table 3 Content of phytosterols in leaves from pot-grown plants and multiple shoot clusters of *C. nutans* cultured in media supplemented with growth regulators for 4 months.

Growth regulator (mg/l)	Phytosterols in multiple shoot clusters (mg/g DW)[1]		
	Lupeol	Stigmasterol	β-sitosterol
Leaves from pot-grown plants	0.04 ± 0.00^{g}	1.56 ± 0.03^{a}	1.76 ± 0.06^{a}
0 TDZ 0 NAA	1.76 ± 0.08^{de}	0.78 ± 0.03^{c}	0.98 ± 0.04^{cd}
0 TDZ 0.01 NAA	1.43 ± 0.04^{e}	0.76 ± 0.01^{c}	0.94 ± 0.01^{cd}
0 TDZ 0.1 NAA	2.47 ± 0.04^{c}	1.02 ± 0.04^{b}	1.16 ± 0.06^{b}
0.5 TDZ 0 NAA	1.96 ± 0.07^{d}	0.48 ± 0.04^{e}	1.07 ± 0.08^{bc}
0.5 TDZ 0.01 NAA	3.19 ± 0.20^{b}	0.50 ± 0.03^{de}	1.08 ± 0.08^{bc}
0.5 TDZ 0.1 NAA	3.93 ± 0.58^{a}	0.44 ± 0.07^{e}	0.91 ± 0.10^{cd}
1 TDZ 0 NAA	0.95 ± 0.23^{f}	0.36 ± 0.10^{f}	0.81 ± 0.20^{d}
1 TDZ 0.01 NAA	2.15 ± 0.13^{cd}	0.26 ± 0.01^{g}	0.64 ± 0.04^{e}
1 TDZ 0.1 NAA	1.87 ± 0.04^{d}	0.56 ± 0.01^{d}	0.92 ± 0.11^{cd}

[1] Means ± standard deviation of three replicates ($n = 3$) within each column followed by the same lowercase superscript are not significantly different according to Duncan's multiple range test ($p \leqslant 0.05$).

highest at 1.56 and 1.76 mg/g DW, respectively, in the leaves from the pot-grown plants, being more than in the multiple shoots from the tissue culture. The amounts of the three kinds of sterol in the tissues cultured in each treatment were significantly different (Table 3).

This result strongly indicated that the combination of TDZ and NAA, which are plant growth regulators (PGRs), induced the production of lupeol in the multiple shoot clusters of *C. nutans*. PGRs not only control fundamental growth and developmental processes but have been used as elicitors to stimulate the production of plant secondary metabolites in plant tissue culture [41]. These chemicals affected the level of genes involved in phytosterol biosynthesis pathway [42]. There has been an increasing trend in secondary metabolite production from *in vitro* plant tissue cultures, revealing many techniques of *in vitro* cell and organ culture for valuable metabolite production [43]. For example, Gonçalves et al [44] reported that *in vitro* shoot-cultures of *Lavandula viridis* had greater amounts of some monoterpenes compared to the field-grown mother-plant. Wongwicha et al [35] reported that the utilization of TDZ with licorice callus was able to stimulate the production of glycyrrhizin. Kitisripanya et al [45] studied callus and cell suspension culture of *Stephania venosa*, with the addition of TDZ 0.5 mg/l and NAA 1.0 mg/l resulting in the cell suspension culture producing 5-folds to 35-folds higher dicentrine contents than from the natural leaf and tuber, respectively.

CONCLUSION

The effect of plant growth regulators on stimulating the production of important bioactive compounds in *C. nutans* or "Phaya Yo" tissue using 0–1.0 mg/l TDZ and 0–0.1 mg/l NAA was studied. This research successfully increased the production of bioactive compounds, such as total phenolic compounds, antioxidant activity and sterol groups and especially, the lupeol content. Multiple shoots cluster from MS medium supplemented with TDZ 0.5 mg/l and NAA 0.1 mg/l stimulated the production of lupeol in tissues 98.25-folds higher than in the leaves from the pot-grown plants. This present work could be recommended as an alternative source for higher production of phenolic substances and lupeol in *C. nutans*.

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