



PHYTOCHEMICAL PRODUCTION ENHANCEMENT ON *IN VITRO* CULTURE OF *Clinacanthus nutans*

(Peningkatan Pengeluaran Fitokimia Pada Kultur *In Vitro* *Clinacanthus nutans*)

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Received: 17 September 2023; Accepted: 2 June 2024; Published: 27 August 2024

Abstract

This study assessed the effects of 6-Benzylaminopurine and wood vinegar on *in vitro* shoot multiplication, and as elicitors in enhancing the phytochemical content of *Clinacanthus nutans* extract. A nodal explant of *C. nutans* was cultured *in vitro* with single or combination treatments in MS medium supplemented with 6-Benzylaminopurine (BAP) or wood vinegar (WV). The growth performance of regenerated shoots was documented after eight weeks of culture. The total phenolic content, total flavonoid content, and antioxidant activities of the leaf extracts were also studied. The results demonstrated that all single treatments with BAP successfully regenerated and multiplied shoots and leaves. While in combination treatments, the data revealed that 2 mg/L BAP with 1% WV (B2WV1) medium treatment led to the highest number of shoots and leaves per explant and the highest total phenolic content and antioxidant activities in the leaf extract. This concludes that combining wood vinegar and BAP treatments in the culture medium caused significant shoot and leaf growth and enhanced the production of leaf's secondary metabolites. These findings highlight the potential of 6-Benzylaminopurine and wood vinegar as elicitors to enhance the production of secondary metabolites in *Clinacanthus nutans*, providing valuable insights for further research in plant biotechnology.

Keywords: *Clinacanthus nutans*, 6-Benzylaminopurine, wood vinegar, *in vitro*, elicitor, phytochemicals

Abstrak

Kajian ini menilai potensi 6-Benzilaminopurin dan cuka kayu untuk penambahan jumlah pucuk *in vitro* dan sebagai elisitor dalam meningkatkan kandungan fitokimia ekstrak *Clinacanthus nutans*. Eksplan nod *C. nutans* dibiakkan secara *in vitro* dengan rawatan tunggal atau gabungan dalam medium MS ditambah dengan 6-Benzilaminopurin (BAP) atau cuka kayu (WV). Prestasi pertumbuhan pucuk yang dijana semula telah didokumenkan selepas lapan minggu kultur. Jumlah kandungan fenolik, jumlah kandungan flavonoid, dan aktiviti antioksidan ekstrak daun turut dikaji. Keputusan menunjukkan bahawa semua rawatan tunggal dengan BAP berjaya menjana semula dan membiak pucuk dan daun. Manakala rawatan gabungan menunjukkan bahawa 2 mg/L BAP dengan 1% WV (B2WV1) membawa kepada bilangan pucuk dan daun tertinggi bagi setiap eksplan, serta jumlah kandungan fenolik dan aktiviti antioksidan tertinggi dalam daun. ekstrak. Ini menyimpulkan bahawa menggabungkan cuka kayu dan rawatan BAP dalam medium kultur menyebabkan pertumbuhan pucuk dan daun yang ketara serta meningkatkan pengeluaran metabolit sekunder daun. Penemuan ini menyerlahkan potensi 6-Benzilaminopurin dan cuka kayu sebagai elisitor untuk meningkatkan pengeluaran metabolit sekunder dalam *Clinacanthus nutans*, memberikan pandangan berharga untuk penyelidikan lanjut dalam bioteknologi tumbuhan.

Kata kunci: *Clinacanthus nutans*, 6-Benzilaminopurin, cuka kayu, *in vitro*, elisitor, fitokimia

Introduction

Clinacanthus nutans, a medicinal plant widely distributed in Malaysia, Thailand, Indonesia, and the Philippines, has traditionally been valued for its potential antioxidant properties [1]. This plant species contains antioxidant phytochemicals crucial in neutralizing free radicals and preventing cellular damage [2]. Antioxidants are indispensable in the body as they neutralize harmful free radicals, safeguard cells against oxidative damage, and reduce the risk of chronic diseases. Free radicals are associated with various severe conditions such as Parkinson's disease, Alzheimer's disease, atherosclerosis, heart attacks, and chronic fatigue syndrome [3]. External antioxidants can aid the body in efficiently scavenging free radicals. Currently, there is significant interest in antioxidants, particularly those that can counteract the anticipated harmful effects of free radicals in the human body and prevent the degradation of lipids and other food components [4]. Synthetic antioxidants like butylated hydroxyl toluene (BHT), butylated hydroxyl anisole (BHA), tertiary butylated hydroquinone (TBHQ), and gallic acid esters were commonly used in the past century. However, their usage has been restricted due to concerns about potential severe health effects [5]. Given the health risks associated with synthetic antioxidants, there is a growing interest in developing natural-based antioxidants from plant sources. Overall, antioxidants derived from natural sources are preferred over synthetic ones [6, 7].

Secondary metabolites are compounds that do not directly contribute to plant growth, development, or reproduction. However, they play crucial roles in environmental interactions, particularly in defense against herbivores and viruses. Examples of these bioactive molecules include alkaloids, flavonoids, terpenoids, and other chemical compounds [8]. Phenolics, as secondary metabolites, are typically present in limited quantities within plant organisms, and their biosynthesis is influenced by various stress factors, both biotic (such as pathogen-induced damage) and abiotic (such as drought, extreme temperatures, and nutrient deficiencies) [9]. Consequently, plant *in vitro* culture provides a reliable system for inducing phenolic accumulation under controlled stress conditions [10]. *In*

vitro culture, also known as micropropagation, involves growing and maintaining plant cells, tissues, or organs under aseptic conditions in a controlled environment. This technique facilitates the rapid multiplication of plantlets, allowing for the mass production of genetically identical plants in a short timeframe [11].

The tissue culture technique significantly enhances shoot regeneration and plant growth rates compared to traditional soil-based methods. However, some plants cultivated in culture media still exhibit low shoot regeneration rates due to inadequate nutrient absorption [12]. By employing *in vitro* selection and manipulating growth regulators, enhancing specific desirable traits in propagated plants becomes feasible. Tissue culture methodologies have been extensively utilized to augment the production of phytochemicals in plant species. The manipulation of culture conditions and specific growth regulators can stimulate the production of desired phytochemicals [13]. Nevertheless, the outcomes of tissue culture can vary based on the specific culture conditions employed. The biosynthesis of target phytochemicals can be prompted by adjusting the type and concentration of growth regulators and incorporating elicitors and other additives [14]. 6-Benzylaminopurine (BAP) is a cytokinin, a plant hormone crucial for regulating various physiological processes such as cell division, differentiation, and development. BAP fosters cell division and proliferation in *in vitro* cultivated plant tissues, leading to increased biomass. Additionally, BAP has been shown to influence the biosynthesis of secondary metabolites in plants [15].

Elicitation involves the application of specific chemical or physical agents to tissue cultures to stimulate the production of secondary metabolites. Elicitors may include substances like jasmonic acid, salicylic acid, or chitosan. Treatment with elicitors mimics the plant's natural defense mechanisms, promoting increased synthesis of phytochemicals [16]. Plants respond to various stressors, such as elicitors, by eliciting an immune response, triggering stress signaling pathways to produce a range of defense compounds collectively known as secondary metabolites [17]. These plant defense metabolites, such as phenolic acids, flavonoids,

and alkaloids, hold medicinal and commercial significance as health-promoting compounds [18].

Wood vinegar, or pyroligneous acid or liquid smoke, is a liquid produced from the destructive distillation of wood or biomass. It contains various compounds, such as acetic acid, butyric acid, catechol, and phenol [19]. Wood vinegar (WV) has been used for various purposes, including as a natural pesticide, fertilizer, and soil conditioner [20]. Wood vinegar contains organic compounds that can influence plant growth and development positively. These compounds act as natural plant growth regulators, promoting root growth, enhancing soil microbial activity, and improving plant nutrient uptake [21, 22]. These properties make wood vinegar an attractive alternative to synthetic growth promoters or elicitors. The use of wood vinegar as an elicitor in plant tissue culture and micropropagation offers several advantages and aligns with current trends in sustainable agriculture and plant biotechnology. Wood vinegar is a natural byproduct of wood processing and biomass decomposition, making it a sustainable and eco-friendly option for agricultural applications [23]. Previous studies have indicated that wood vinegar can stimulate callus formation, shoot proliferation, and root initiation in various plant species [24, 25]. WV contains natural plant growth regulators that can influence plant growth and development positively [19]. Unlike synthetic growth regulators or chemical elicitors, wood vinegar is derived from renewable resources and does not pose environmental risks associated with synthetic compounds.

Additionally, wood vinegar's ability to enhance nutrient availability and microbial activity in the soil can indirectly contribute to improved plant health and secondary metabolite production [26, 27]. In addition, wood vinegar has natural pesticidal properties, and its application can deter certain pests and diseases that may otherwise hinder plant growth [19]. Its phenolic compounds can stimulate the plant's own defense mechanisms by triggering the production of phytoalexins, which are antimicrobial substances produced by plants in response to pathogen attack. This strengthens the plant's ability to resist infections and enhances its overall resilience by increasing the secondary metabolites production [28]. Utilizing wood vinegar as an elicitor in tissue culture and micropropagation can be cost-effective compared to

synthetic growth regulators or specialized elicitors [29]. Agricultural waste or biomass-derived products like wood vinegar offer a sustainable and economically viable alternative for enhancing plant growth [19] and phytochemical production [30, 31]. However, WV's complex composition requires careful dosage optimization for each application to maximize its effectiveness due to potential synergistic or antagonistic interactions among its constituents [32]. Therefore, optimizing WV administration is crucial to understanding its mechanism as an elicitor of plant bioactive compounds, as demonstrated here for an economically important crop like *C. nutans*.

As reported in previous research by Hashim et al. [7], an evaluation of phytochemical constituents across different parts of *C. nutans* revealed higher values of Total Phenolic Content (TPC) at 228.06 mg GAE/g DW, Total Flavonoid Content (TFC) at 29.76 mg QE/g DW, and quercetin at 0.021 g/g in intact leaf extracts. These findings also highlighted the superior antioxidant properties of intact *C. nutans*, with DPPH scavenging activity recorded at 15.50% and FRAP values at 15.68 FeSO₄/g. Notably, previous successful studies employing elicitors were conducted exclusively on intact *C. nutans* plants, such as the work by Mohan [33] using short-term heat stress as an elicitor, which significantly increased rutin, quercetin, and kaempferol production. However, there has been limited exploration of phytochemical values in *C. nutans in vitro* cultured plants, a little less on evaluating the effect of elicitor towards enhancement of plant secondary metabolites within the *in vitro* cultured plant. Therefore, there is a need for a preliminary study to *in vitro* culture this plant and screen its phytochemical constituents.

Combining BAP with other additional supplements will synergistically enhance the propagation process, as demonstrated in a previous study where kinetin, a cytokinin plant hormone, was used alongside BAP to achieve high rates of multiple shoot induction [34]. It is important to note that the impact of wood vinegar and BAP hormone on plant micropropagation can vary based on factors, specifically its application in the growth medium. Hence, optimization is crucial to determine the ideal application and concentration levels for promoting plant growth and maximizing the production of phenolic and flavonoid compounds. The present study aimed to develop an easy and efficient protocol for direct shoot

regeneration of *C. nutans* in medium with the addition of BAP or WV in single or by combination. Subsequently, the regenerated plants were subjected to different biochemical analytical assays through antioxidant enzyme activities, total phenolic and flavonoid content (TPC and TFC) determination, and free radical scavenging activity.

Materials and Methods

Wood vinegar preparation

The wood vinegar used in this agricultural study was sourced from a reputable supplier in Kuala Sepetang, Taiping, Perak, Malaysia. It was certified to be at least 95% pure, with an acetic acid concentration between 1% and 5%. The solution underwent filtration using Whatman No. 1 filter paper to remove debris, oily phases, or suspended particles, followed by autoclaving and storage at 4°C until further use.

Media preparation for shoot regeneration

Murashige and Skoog (MS) medium were used for shoot regeneration. MS powder at 0.44 % (w/v) was dissolved in distilled water with 3% (w/v) of sucrose. The 6-Benzylaminopurine (BAP) or WV was added in a single or combination medium at different concentrations in preparation for nine different medium treatments. Gelrite (7 g) was dissolved in the solution for 1 liter of medium final volume. The culture medium was autoclaved for 15 minutes at 121 °C and 15 psi. Single treatments were prepared individually by adding 6-Benzylaminopurine (BAP) or wood vinegar (WV) to the culture medium. Different concentrations of BAP (0.5, 1, and 2 mg/L) and WV (1, 2, and 4 %) were used in separate batches to assess their effects on plant tissue cultures. On the other hand, the combination treatments were prepared by adding a fixed concentration of 2 mg/L BAP to the culture medium. Various concentrations of wood vinegar (WV) ranging from 1, 2, and 4% were combined with the BAP-containing medium. The 2 mg/L of BAP hormone was used based on optimization studies conducted during preliminary testing that yielded the most favorable outcomes in promoting plant growth and development. Control groups were included in the experimental setup and served as baseline references for comparison against the single and combination treatments.

Establishment of aseptic culture

The sterilization step was according to Hashim et al. [1]. The nodal explants were excised from the plant and cleaned adequately under running tap water for five minutes. Explants were prewashed for one minute with 70% (v/v) ethanol and then rinsed thrice with sterile filtered water. Next, explants were dipped inside 0.1% HgCl₂ for 30 minutes and rinsed thrice for 5 minutes each. The sterile explant was then air-dried.

Shoot regeneration

The explants were inoculated vertically on MS medium for culture initiation. The cultures were incubated at 25 ± 2 °C under light (40 μmol m⁻² s⁻¹ Photosynthetic Photon Flux Density (PPFD) for 25–30 days. The growth performances of successfully regenerated shoots and leaves were measured in week eight by calculating the number of shoots per explant, shoot length, number of leaves per explant, and leaf length. The shoot and leaf length were measured by the unit of cm. The regenerated shoots were then sub cultured once every four weeks in a new medium.

Extraction

The regenerated plant was oven-dried (60°C, 24 h), and the extraction was performed according to the protocol described by Ali et al. [35] with minor modifications. Each finely ground sample (100 mg) was mixed with methanol (10 ml). The mixtures were sonicated (10 min) 3 times with a resting period of 30 min in between and centrifuge (8000 rpm, 10 min). The supernatants were collected and stored at 4° C.

Total phenolics and flavonoids determination

The total phenolic content of samples was determined using Folin-Ciocalteu reagent and gallic acid as standard, according to Singleton and Rossi [36], with minor modifications. Briefly, 1 mg of dried crude extract was dissolved in 1 mL methanol. Next, 0.2 ml of the mixture was added with 0.6 mL of water and 0.2 mL of Folin-Ciocalteu's phenol reagent. After 5 min, 1 mL of saturated sodium carbonate solution (8% w/v) was added to the mixture, and the volume was made up to 3 mL with distilled water. The reaction was kept in the dark for 30 min. After centrifuging, the absorbance of extracts was measured using a visible spectrophotometer at 765 nm against a reagent blank without the extract. A stock solution of gallic acid (500 mg/L) was prepared in

distilled water and diluted to appropriate concentrations (5–500 mg/L) to construct the calibration curve. The total phenol concentration in samples was measured using the calibration equation ($y = 0.0024x$, $r^2 = 0.9896$) and expressed as μg of gallic acid equivalent (GAE) per g dry weight. All assays were carried out in triplicate.

The aluminum chloride colorimetric method was used to determine the total flavonoid content of the sample. Quercetin was used to make the standard calibration curve for total flavonoid determination. The standard solutions of quercetin were prepared by serial dilutions using methanol (5–200 mg/L). An amount of 0.6 mL diluted standard quercetin solutions or extracts was separately mixed with 0.6 mL of 2% aluminum chloride. After mixing, the solution was incubated for 60 min at room temperature. The absorbance of the reaction mixtures was measured against a blank at 420 nm wavelength with a Varian UV-Vis spectrophotometer. The concentration of total flavonoid content in the test samples was calculated from the standard curve equation ($y = 0.0153x$, $r^2 = 0.9615$), and results were expressed as mg quercetin equivalent (QE)/g of dried plant material. All the determinations were carried out in triplicate.

Antioxidant analyses

The DPPH free radical scavenging assay described by Gabr et al. [21] was used for antioxidant activity determination. The stock reagent solution (1 mM) was prepared by dissolving 22 mg of DPPH in 50 ml of methanol and stored at 20 °C until use. The working solution (0.06 mM) was prepared by mixing 6 ml of stock solution with 100 ml of methanol. Extract samples (1mg/ml) and synthetic antioxidant (BHA) solutions (0.1 ml of each) were vortexed for 30 s with 3.9 ml of DPPH working solution. After a 30-minute incubation period at room temperature in the dark, absorbance was recorded at 515 nm using a spectrophotometer. The DPPH solution without extract served as a control. The scavenging activity was calculated as follows:

$$\% \text{ inhibition} = \frac{[\text{absorbance of control} - \text{absorbance of sample}]}{\text{absorbance of control}} \times 100 \quad (1)$$

Ferric Reducing Antioxidant Power (FRAP) assay was carried out according to the method of Wee [22]. FRAP

reagent was freshly prepared by mixing 300 mM sodium acetate (pH 3.6), 10 mM 2,4,6-tripyridyl-1,3,5-triazine (TPTZ) dissolved in 40 mM of HCl, and 10 mM iron (III) chloride hexahydrate ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$) in a ratio of 10:1:1. A total of 200 μl of extracts was gently mixed with 3 ml of FRAP reagent and incubated at 37°C in water bath for 30 minutes. The increase in absorbance at 593 nm was measured after 30 minutes against the FRAP reagent blank. Standards of known FeSO_4 (0-500 μmol) were used to generate a calibration curve ($y = 0.0025x + 0.05$, $R^2 = 0.9972$), and the results were expressed as mg FeSO_4 equivalent per g dry weight samples (DW).

Statistical analysis

Each sample for the analytical test was carried out in triplicate, and the data was presented as average $\pm$ SE of the three-absorbance reading. The data was analyzed by the Analysis of variance (ANOVA) followed by Duncan's multiple range test. The data analysis was performed using SPSS version 20.

Results and Discussion

Shoot growth performance

A comparative analysis was conducted between single and combined treatments involving 6-Benzylaminopurine (BAP) and wood vinegar (WV) to assess their efficacy in establishing cultures. The study discerned that including BAP hormone in the single treatment medium notably stimulated shoot growth, with the optimum promotion observed at 2 mg/L BAP, resulting in 2.77 shoots per explant (refer to Table 1). Conversely, when using the MS medium with a sole addition of wood vinegar (WV), successful initiation and significant promotion of shoots were achieved. Specifically, the WV1 medium (1% WV) successfully initiated 2.64 shoots per explant (refer to Table 1). Moreover, the combination treatment involving 2 mg/L BAP and 1% WV (B2WV1) yielded higher plant shoot numbers and lengths than single treatments. The MS medium supplemented with B2WV1 exhibited the maximum number of shoots per explant (10.60), showcasing its superior efficacy in promoting shoot proliferation. Additionally, this supplemented medium demonstrated notable shoot length, measuring 2.51 cm, as indicated in Table 1.

Table 1. Regenerated plant growth performance after 8 weeks of culture

Code	Number of Shoots Per Explant	Length of Shoots (cm)	Number of Leaves Per Explant	Length of Leaves (cm)
CS	1.33 ± 0.03 ^h	0.72 ± 0.01 ^e	2.70 ± 0.02 ⁱ	0.64 ± 0.02 ^d
<i>Single</i>				
B0.5	1.30 ± 0.02 ⁱ	0.63 ± 0.02 ^f	2.77 ± 0.01 ^h	0.60 ± 0.04 ^e
B1	1.63 ± 0.02 ^g	0.89 ± 0.02 ^d	3.53 ± 0.02 ^f	0.90 ± 0.02 ^c
B2	2.77 ± 0.01 ^c	1.90 ± 0.06 ^b	4.17 ± 0.02 ^c	1.59 ± 0.02 ^a
WV1	2.64 ± 0.01 ^d	0.46 ± 0.04 ^h	3.91 ± 0.02 ^k	0.36 ± 0.02 ^f
WV2	0.00 ^j	0.00 ^j	0.00 ^k	0.00 ^h
WV4	0.00 ^j	0.00 ^j	0.00 ^k	0.00 ^h
<i>Combination</i>				
B2WV1	10.60 ± 0.03 ^a	2.51 ± 0.05 ^a	9.30 ± 0.01 ^a	0.56 ± 0.01 ^e
B2WV2	2.17 ± 0.03 ^f	0.24 ± 0.02 ⁱ	1.33 ± 0.01 ^j	0.39 ± 0.03 ^f
B2WV4	0.00 ^j	0.00 ^j	0.00 ^k	0.00 ^h

(CS: control; B0.5: 0.5 mg/L BAP; B1: 1 mg/L BAP; B2: 2 mg/L BAP; WV1: 1% WV; WV2: 2% WV; WV4: 4% WV; B2WV1: 2 mg/L BAP + 1 % WV; B2WV2: 2 mg/L BAP + 2 % WV; B2WV4: 2 mg/L BAP + 4 % WV). Values (separate significance for each parameter) with the same superscripts were not found to be significantly different from each other.

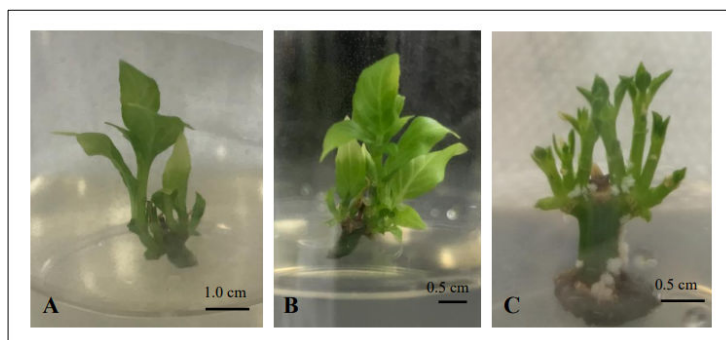


Figure 1. Development of regenerated plant from nodal explant culture in medium supplemented with (A-B) 2mg/L BAP (B2) (C) 2 mg/L BAP + 1 % WV (B2WV1)

Leaf numbers serve as a critical parameter for evaluating the *in vitro* growth of explants, particularly concerning their developmental progression. Data revealed a significant impact of the WV and BAP addition on leaf production in regenerated shoots. An optimal leaf count was observed in the combination treatment. Specifically, the MS medium supplemented with 2 mg/L BAP and 1% WV (B2WV1) demonstrated the highest leaf production, averaging 9.30 leaves per explant. However, increasing the WV concentration to 2% (B2WV2) resulted in a decline in leaf number to 1.33 leaves per explant. Moreover, although the leaf number peaked in the B2WV1 treatment, the leaf size was relatively small, averaging 0.56 cm per leaf. In contrast, a sole treatment of 2 mg/L BAP in the MS medium yielded the longest leaves, averaging 1.59 cm in length. Figure 1 visually depicts the development of adventitious shoots from

nodal explants of *C. nutans* under single (B2) and combination (B2WV1) medium treatments. Notably, the B2 treatment showed robust growth of leafy shoots, while B2WV1 exhibited distinctive adventitious shoots with smaller leaf sizes.

This study demonstrates the substantial positive impact of synergistically combining wood vinegar (WV) and the BAP hormone to enhance plant growth. It highlights their potential as advantageous supplements in tissue culture applications. Previous studies have identified acids and phenols as crucial wood vinegar constituents, exhibited significant biological activity and promoted plant growth [37]. WV is acknowledged for its capacity to improve crop yield, bolster plant health, and elevate harvest quality [19]. The organic acids, phenols, and other organic compounds found in wood vinegar (WV)

act as typical allelochemicals that regulate plant growth by influencing ion absorption, respiratory metabolism, hormone equilibrium, and protein synthesis [27]. The exogenous application of phenolic compounds in plants plays a crucial role in enhancing plant growth and mitigating the effects of abiotic and biotic stress through mechanisms such as promoting the lignification of plant cell walls, which enhances shoot length [38]. This observation aligns with the findings of Ofoe et al. [39], who highlighted that the synergistic effect of the combined foliar application of WV and soil fertilizer contributed to increasing tomato leaves' growth performance.

In addition, the growth of regenerated shoots and leaves in the combination treatment is significantly influenced by the concentrations of wood vinegar (WV). High concentrations of WV exert an inhibitory effect on plant growth, leading to a decline in shoot production with further increases in WV concentration (2% and 4%) in the combination treatment. The detrimental effects of high WV concentrations can be attributed to elevated medium acidity levels, which are unfavorable for plant development and can induce phytotoxicity, acting as stressors and potentially functioning as herbicides [31]. Acidic conditions can also adversely affect plant growth by damaging cell structures and suppressing growth responses [40]. Furthermore, specific components in wood vinegar may interfere with nutrient uptake or disrupt physiological processes within plants, potentially leading to reduced leaf size or altered growth patterns. Therefore, adding WV at an appropriate concentration is crucial to optimize shoot growth [19].

This study suggests that moderate concentrations of WV, when used in conjunction with BAP, create a conducive environment for plant growth by enhancing the activity of key enzymes involved in metabolic pathways related to stress response and secondary metabolite production. This study highlights the potential of combining WV and BAP hormone as a synergistic treatment to enhance plant growth and resilience. The organic compounds in WV, particularly when used at optimal concentrations, can significantly improve plant health and productivity. Future research

should focus on elucidating the precise molecular mechanisms underlying these synergistic effects and exploring the long-term implications of such treatments. Additionally, further studies could investigate the potential application of this combination in different plant species and under various environmental conditions to generalize the findings and develop comprehensive guidelines for practical use in agriculture and tissue culture. By understanding and optimizing the concentrations and conditions for applying WV and BAP, their full potential could be harnessed to improve plant growth and yield, paving the way for more sustainable and efficient agricultural practices.

Effect of hormonal and WV treatment on polyphenols content of *in vitro* cultured leaf extract

Figure 2 illustrates the varying phenolic compound concentrations in different treatments. Applying BAP and WV has significantly elevated Total Phenolic Content (TPC) in *C. nutans*. Specifically, 1% WV (WV1) increased TPC from 23.68 to 77.45 mg GAE/g DW, representing a 3.27 times increment. Meanwhile, 2 mg/L BAP (B2) raised TPC from 23.68 mg GAE/g DW to 121.95 mg GAE/g DW, marking a more increment given 5.15 times. Furthermore, the combined treatment of 2 mg/L BAP and 1% WV (B2WV1) exhibited the highest phenolic increment, reaching 219.75 mg GAE/g DW from the initial 23.68 mg/g (control) given 9.27 times increase.

A slightly different trend was observed in the total flavonoid content of the extract. The highest total flavonoid content (56.60 mg QE/g DW) was noted with the application of 2 mg/L BAP (B2), representing 5.25 times increase. Following this, B2WV1 displayed 35.95 mg QE/g DW, 3.27 times increase compared to the control's 10.99 mg QE/g DW (Figure 2). These results indicated that supplementation of both BAP and WV in MS medium has a stimulatory effect on the accumulation of phenolics and flavonoids in the regenerated plant of *C. nutans*. This phenomenon can be attributed to the stimulating effects of BAP on secondary metabolism and the bioactive attributes of WV components.

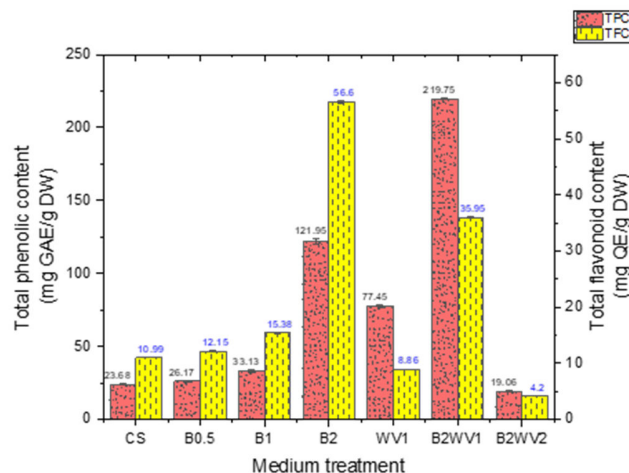


Figure 2. Total phenolic and flavonoid compounds of regenerated shoots of *C. nutans* from different medium treatments

Exogenous application of BAP hormone at high concentration has been shown to enhance the yield and medicinal quality of *C. nutans* by promoting the accumulation of phenolics and flavonoids. Due to oxidative stress in target cells, the elicitor stimulates the plant to increase immune responses and PAL gene expression, leading to an increase in secondary metabolites, especially phenolic compounds, which are part of the systemic acquired resistance process [41]. Zhang et al. [42] demonstrated that BAP can upregulate gene expression levels, particularly PAL, 4CL, CHS, and F3GT, crucial enzymes in the flavonoid synthesis pathway. Baskaran et al. [43] reported that the application of BAP in media resulted in elevated levels of phenolic and flavonoid content in the shoot cultures of *Coleonema pulchellum*. Enhancement in the PAL activity in response to exogenously applied TDZ and BA is linked to the stimulating effects of these PGRs on the regulation of the PAL gene at the transcriptional level [44].

Furthermore, the elevated levels of phenolics and flavonoids in *C. nutans* following BAP treatment can be attributed to the inclusion of Wood vinegar (WV). In this investigation, WV acts as an elicitor by effectively enhancing the production of secondary metabolites, mainly phenolic compounds and organic acids. The phenols derived from Wood vinegar (WV) are pivotal in mitigating plants' abiotic and biotic stress effects through various mechanisms. Phenols prevent

pathogens from infiltrating the host plant and influence the activity of specific enzymes like antioxidant enzymes and the synthesis of compounds such as proline and phenolic compounds [45]. Additionally, acetic acid in WV may activate plant defense gene regulators. This aligns with prior research indicating that acetic acid in the medium successfully boosted the production of plant secondary metabolites [46, 47, 48, 49].

The current study reveals that combining 6-Benzylaminopurine (BAP) hormone and wood vinegar generates a synergistic effect, enhancing stress response and triggering increased production of secondary metabolites. The medium treatment influences the activity of enzymes involved in flavonoid and phenol biosynthesis. Earlier research has shown that Karrikin present in WV function similarly to other phytohormones, interacting with gibberellins, ethylene, and cytokinin to regulate plant growth and productivity [50, 39]. This suggests that WV may have induced and collaborated with enzymes and BAP hormones to increase plant yield. Integrating BAP with WV not only enhances the accumulation of beneficial secondary metabolites but also suggests a practical approach for improving plant resilience and productivity.

The DPPH and FRAP assay (Figure 3) indicate significant differences in the ability to scavenge free radicals among all medium treatments. The plant grown in medium with 2 mg/L BAP and 1% WV (B2WV1)

extract exhibited a more potent antioxidant activity (DPPH = 5.59 % and FRAP = 25.46 g FeSO₄/g DW) than 2 mg/L BAP (B2) given DPPH = 2.42 % and FRAP = 12.37 g FeSO₄/g DW. All extracts could directly react

and quench the DPPH and Fe³⁺ radicals. The combination treatment of BAP and WV (B2WV1) exhibited the highest activity when compared with a single treatment of BAP or WV individually.

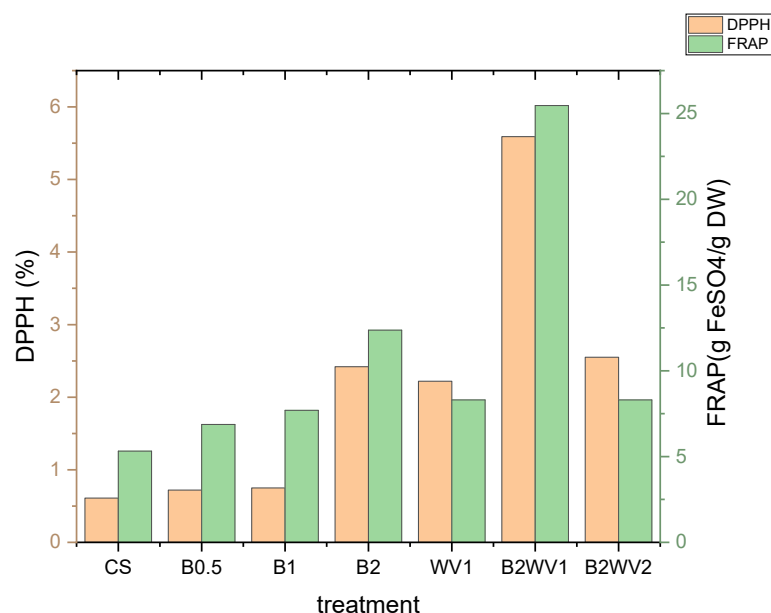


Figure 3. DPPH and FRAP activity of regenerated plant of *C. nutans* that cultured on MS medium comprising BAP and WV

Correlation coefficient of TPC, TFC, and DPPH and FRAP assay is given in Table 2. Results showed that TPC is highly correlated with DPPH and FRAP assay ($R^2 = 0.991$ and $R^2 = 0.929$). This means that TPC is highly contributed to the antioxidant capacities of *C.*

nutans extracts more than the correlation coefficient with TFC towards DPPH and FRAP assay ($R^2 = 0.258$ and $R^2 = 0.312$), which might partially be due to their lower antioxidant activity (Fig. 2c).

Table 2. Correlation between measured parameters of the regenerated plant of *C. nutans*

	TFC	DPPH	FRAP
TPC	0.307*	0.991*	0.929*
TFC		0.258*	0.312*
DPPH			0.890*

Correlation is significant at the 0.05 level with the label (*).

The positive correlations between phenolic and flavonoid levels elucidate their role in enhancing the antioxidant potential of the extract. This study reinforces the findings of Ghasemzadeh et al. [51], Khoo et al. [52], and Baharuddin et al. [53], where the robust antioxidant activity of *C. nutans* extract was attributed to its phenolic and flavonoid content. Phenolics and flavonoids are recognized for their antioxidant properties, facilitated by

their hydroxyl groups that contribute to radical scavenging capabilities [54]. Kapoor et al. [55] also reported a positive correlation analysis between Total Phenolic Content (TPC) and DPPH free radical scavenging activity in *R. imbricata* callus cultures exposed to various light spectra. Similar positive correlations have been observed between phenolics,

flavonoids, and antioxidant activity in diverse medicinal plants like *Cichorium pumilum* [56].

Incorporation of BAP and WV into MS basal media not only regulated the *in vitro* morphogenesis processes, leading to direct shoot regeneration in *C. nutans*, but also influenced the production of phenolic and flavonoid compounds. The *in vitro* synthesis of these phytochemical constituents is intricately governed by direct shoot induction from the cultured explants. The varying effects of BAP and WV treatments on the production of these beneficial compounds can be ascribed to several factors that impact *in vitro* shoot regeneration in *C. nutans*, such as the maintenance of the regenerated shoot phase *in vitro* and the defense mechanisms of plant tissue against supplemented growth regulators [57]. By understanding and optimizing the concentrations and conditions for applying WV and BAP, their full potential could be harnessed to improve plant growth and yield, paving the way for more sustainable and efficient agricultural practices. Future research should focus on elucidating the precise molecular mechanisms underlying these synergistic effects and exploring the long-term implications of such treatments. Additionally, further studies could investigate the potential application of this combination in different plant species and under various environmental conditions to generalize the findings and develop comprehensive guidelines for practical use in agriculture and tissue culture.

Conclusion

In conclusion, it was determined that *C. nutans* has good plant regeneration potential from nodal explant in MS medium supplemented with BAP hormone and wood vinegar. The highest number of shoots and leaves per explant was found on MS media supplemented with 2 mg/L BAP and 1% WV. Also, the present study showed that adding BAP and WV gives the highest elicitation effect on promoting the production of total phenolic and flavonoids. The antioxidant activities in *C. nutans* regenerated plant extract are also highly correlated with the total phenolic content. This study showed that adding BAP and WV, especially in combination treatment for promoting the *C. nutans* plant regeneration, also enhances the content of pharmacologically valuable metabolites in *C. nutans*.

Acknowledgement

The authors are most grateful to the Faculty of Applied Sciences, UiTM Perak branch, Tapah campus, for laboratory and facilities support.

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