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# In Vitro Germination, Callus Induction and Shoot Multiplication of Important Medicinal Plant, Christia Vespertilionis (L.F.) Bakh.F.

**[1,2] Shahril Efzueni Rozali, [2]Mahanom Jalil, [2]Nur Kusaira Khairul Ikram, [2]Jamilah Syafawati Yaacob, [1]Lim Yen-Theng, [1] Sakthivel Balakrisnan**

[1] Faculty of Science, Technology, Engineering and Mathematics, International University of Malaya-Wales, 50480 Kuala Lumpur, Malaysia,

[2] Institute of Biological Sciences, Faculty of Science, University of Malaya, 50603 Kuala Lumpur, Malaysia

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***Abstract:***
Christia vespertilionis (L. f.) Bakh. f. is an important medicinal plant belongs to Fabaceae family that plant commonly known as butterfly wing or 'rerama'. It has been used in traditional medicine and was reported to have anticancer, anti-inflammation and antiplasmodium activity. In vitro culture technique potentially can enhance the propagation and bioactive compounds production from this unexploited medicinal plant. This study established the in vitro cultures of C. vespertilionis using seed explants and aseptic seedlings. Four different pre-treatments were used to induce the in vitro germination consist of 50% (v/v) HCl, 0.2% (w/v) $KNO_3$, 0.3 g/L $GA_3$ and distilled water as control. Seeds treated with 50% (v/v) HCl showed the highest percentage of germination. Induction of callus and shoot multiplication was initiated from aseptic seedlings in MS medium supplemented with different concentration of 6-Benzylaminopurine (BAP) in combination with 1-Naphthaleneacetic acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D). Explants of leaf, petiole and stem were produced different colour of callus consist of green, brownish green and yellowish green. The highest callus and multiple shoot production were observed in stem explant cultured on MS medium augmented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D. Green callus was only produced in medium supplemented with NAA whereas green and brownish green callus was observed in medium supplemented with 2,4-D. Compact callus was produced in MS medium with combination of 1.0 mg/L BAP and 1.0 mg/L NAA while most friable callus were produced in MS medium supplemented with 2,4-D. This study is the first to report on the production of callus and multiple shoots from aseptic seedlings of C. vespertilionis.

***Keywords:*** *Callus, Christia vespertilionis, 2,4-dichlorophenoxyaceticacid., seed germination, shoots multiplication.*

## I. INTRODUCTION

*Christia vespertilionis* (L. f.) Bakh. f. also known as butterfly wing or rerama (Malay) is an important medicinal plant belongs to Fabaceae family. This plant is native to South China and tropical Southeast Asia including Malaysia. This plant was traditionally used to treat various illnesses such as scabies, tuberculosis, colds, muscle weakness, bronchitis and inflamed tonsils [1]. The leaves were dried and mostly commercialized as herbal tea for alternative medicine in several countries due to its potential to treat cancer [2]. Several studies have reported and indicated that *C. vespertilionis* could potentially be used as an anticancer for treatment of various cancer diseases. The antitumor activity of *C. vespertilionis* extracts was demonstrated by [3] against human neuroendocrine tumor cells consist of

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and small intestinal neuroendocrine tumor and medullary thyroid carcinoma. The *C. vespertilionis* extracts also have been tested for *in vivo* antitumor activity against S180 and H22 tumor cells in mice model. The results revealed that the growth of S180 and H22 tumor cells were inhibited by the extract without significant effects in hematopoietic system and immune function of the mice [4]. The combination treatment of ethanolic extract of *C. vespertilionis* and cyclophosphamide was recently reported to have synergism interactions on WRL68 cell lines [5]. *C. vespertilionis* was reported to have antimalarial activity [6]. Two newly identified bioactive compounds were characterized from from the *n*-hexane fraction of *C. vespertilionis* extracts named as christene and christanoate. [1] reported that the novel metabolite christene has potent antiplasmodial activity against *Plasmodium falciparum.* The plant extracts also showed highest suppression (87.8%) of parasitaemia in *P. berghei* infected mice. Furthermore, the safety of subacute oral administration of ethanolic leaf extract of *C. vespertilionis* was tested in rats with no significant lesions in kidney at different dosage [7]. This toxicology analysis is very crucial in order to evaluate the side effects to those people who consume it as an alternative medicine or supplement. The extraction and oil's solubility of *C. vespertilionis* at different temperature range have been reported recently and was determined through green separation technology using supercritical carbon dioxide fluid [8]. This extraction strategy is important to overcome several problems of conventional organic solvent extraction by reducing the impurities, degradation of compound, extensive extraction time and environmental concerns.

Plant cell and tissue cultures represent an alternative strategy for the mass clonal propagation and plant secondary metabolite production for industrial and medical applications. This method provides a sustainable and eco-friendly system to obtain bioactive compounds under controlled and contaminant-free environment which allow the production of desired compounds from various plant species on an industrial scale [9]. Yet despite all of these advantages, there was no report on callogenesis and regeneration of *C. vespertilionis* has been published. The aim of this study is to establish the *in vitro* cultures of *C. vespertilionis,* realizing its huge potential as an alternative medicine for malaria and cancer diseases, and its crucial need of sustainable sources for extraction. The present study evaluated the *in vitro* seed germination as well as production of callus and regeneration in *C. vespertilionis*.

## II. MATERIALS AND METHODS

### *A. Pretreatment of seeds*

Seeds of *C. vespertilionis* were purchased from local nursery located at Sungai Buloh, Malaysia. The seeds were washed with liquid detergent and rinsed using distilled water to remove the debris. The cleaned seeds were subjected to various pretreatments by soaking in different solutions consist of distilled water, gibberellic acid, potassium nitrate and hydrochloric acid at different concentrations and duration (Table 1).

### *B. Germination of seeds*

All treated seeds were collected and transferred into laminar air flow chamber for surface sterilization process. Under aseptic conditions, seeds were washed with 70% (v/v) bleach for 15 minutes followed by 3 times rinse with autoclaved distilled water. After that, seeds were soaked in 0.2% mercury chloride ($HgCl_2$) for 10 minutes. After rinsed 3 times with autoclaved distilled water, seeds were blotted on sterilized filter papers to remove excess moisture. All disinfected seeds were cultured on free hormone Murashige and Skoog (MS) basal medium augmented with 3% (w/v) sucrose and solidified with 0.3% (w/v) Phytagel (Sigma-Aldrich) at pH 5.8 prior to autoclaving. The seeds were incubated at 25°C with 16/8 light dark period. The percentage of germination and number of shoots produced were observed after 30 days of culture.

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*C. Callus induction and regeneration of shoots*

Murashige and Skoog (MS) basal medium were used in this study. The media were supplemented with three different concentrations of 6-benzylaminopurine (BAP) (1.0, 1.5 and 2.0 mg/L) in combination with either 1.0 mg/L 2,4-dichlorophenoxyaceticacid (2,4-D) or 1.0 mg/L 1-naphthaleneacetic acid (NAA) as shown in Table 2 and 3. The media was added with 3% (w/v) sucrose as carbon source and solidified with 0.3% (w/v) Phytagel (Sigma-Aldrich) prior adjusted to pH 5.8 before autoclaved. Three different explants were used to induce the callus formation (callogenesis) and shoot regeneration (organogenesis) consist of petiole, leaf and stem explants (Figure 2). Under aseptic condition, the explants were excised from aseptic seedlings of *C. vespertilionis* at size 0.5 cm length and cultured on the media. All cultures were incubated at 25°C with 16/8 light dark period and observation was recorded every two weeks.

*D. Statistical analysis*

The study was carried out in triplicate cultures with 8 explants for seed germination and 15 explants for callogenesis and organogenesis for each replicate. All collected data were analysed by one-way ANOVA followed by Duncan's Multiple Range Test (DMRT) at a significance level of $P < 0.05$. The data obtained were analysed using SPSS Software version 21.

## III. RESULTS AND DISCUSSION

*A. Effects of different pretreatment on germination of seed.*

Percentage of germination and number of shoots produced between different pretreatments and its duration have been recorded. Percentage of germination varied among different pretreatment methods. Seeds treated with 50% HCl (10 minutes) has the highest percentage of germination (52%) followed by 0.3 g/L $GA_3$ (24 hours) and distilled water (3 hours) with 40% and 37.8% of germination, respectively (Table 1.0). The same response was observed in the production of shoots where pretreatment with 50% HCL (10 minutes) has produced the highest number of shoots (2.41±0.28). However, the number of shoots produced in seeds treated with 0.3 g/L $GA_3$ (24 hours) and distilled water (3 hours) have less significant different with 2.26±0.31 and 2.19±0.27 of shoots, respectively. The overall results showed that prolonging of the pretreatments could increase the percentage of germination in *C. vespertilionis.*

The utilization of HCL for improvement the germination of seeds has been reported in germination of sugar beet and African locust bean [10]-[11]. Pretreatment of seeds with HCL will disrupt and soften the seed coat and allowing the imbibition of water which triggers the germination. The increasing of germination after application of $GA_3$ indicated that seeds of *C. vespertilionis* exhibited an intermediate physiological dormancy due to the present of its seed coat. The same observation was recorded in germination of *Verbascum calycosum* seeds [12]. The exogenous application of $GA_3$ can recover the deficiency of endogenous $GA_3$ to break the seeds dormancy [13]. Seeds treated with 0.2% $KNO_3$ showed the lowest percentage of seeds germination (12%) and number of shoots (1.38±0.30 shoots). These results demonstrated the negative effect of salinity on seeds germination in *C. vespertilionis.* The exposure to salt will increase the salinity level which negatively affect the water uptake by seed and inhibit the germination [12]. The present results also demonstrated that prolonging of pretreatment with HCL and $GA_3$ will enhance the percentage of germination. However, the maximum duration of exposure needs to be determined to avoid the harmful effects on the seeds [14].

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**Table 1**: Effects of different pretreatments on percentage of germination and production of shoots in *C. vespertilionis*.

| Pretreatments | Duration | Germination (%) | Number of Shoots |
|---|---|---|---|
| Distilled water | 3 hours | 37.8 | 2.19 ± 0.27b |
| Distilled water | 24 hours | 20.0 | 1.83 ± 0.29d |
| 0.3 g/L $GA_3$ | 24 hours | 30.0 | 2.05 ± 0.29bc |
| 0.3 g/L $GA_3$ | 48 hours | 40.0 | 2.26 ± 0.31b |
| 0.2% $KNO_3$ | 24 hours | 12.0 | 1.38 ± 0.30e |
| 50% HCl | 5 minutes | 28.0 | 1.97 ± 0.31c |
| 50% HCl | 10 minutes | 52.0 | 2.41 ± 0.28a |

*Mean with the different letters in each column are significantly different by Duncan's Multiple Range Test (DMRT) at $p<0.05$*

**Fig. 1**: Regeneration of *in vitro* seedlings of *C. vespertilionis* in free hormone MS medium.

*B. Induction of callus*

Three types of explants (petiole, stem and leaf) from the *in vitro* seedlings were used to induce the formation of callus. The percentage of callogenesis between different explants and combinations of plant growth regulators were recorded in Table 2.0 with no significant differences. Callus were started to produce in all explants after four weeks of culture. However, some of the stem and leaf explants cultured on MS medium with BAP and NAA have no callus formation and turned brown after six weeks of culture. In general, MS medium supplemented with 2,4-D showed better results in production of callus with 100% response in all types of explants. The medium composition and its formulation were considered as important factors in enhancing the formation of callus [9]. Combination of 2,4-D with other cytokinins have showed a better result in formation of callus due to the increasing of catalytic activity of the kinases caused by both plant growth regulators which induce the cells to undergo division [15].

In the present study, petiole explants produced more compact callus while a mixture of friable and compact callus were observed in leaf and stem explants (Table 3.0). Green callus was produced on MS medium supplemented with BAP and NAA whereas yellowish green callus was produced on MS medium supplemented with BAP and 2,4-D. The pigmentation of callus affected by different concentration and combination of plant growth regulators [16]. Furthermore, the increasing of concentration of BAP in combination with 2,4-D caused the leaf explants turned brown with less production of callus. The browning of the callus was due to the toxicity effect of high concentration of cytokinins [9].

The biomass production of callus was significantly affected by different combinations and concentrations of plant growth regulators as shown in Table 4.0 and Fig. 2. Overall, MS medium augmented with BAP and 2,4-D showed better results compared to combinations of BAP and NAA. Stem explants cultured on MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D showed the highest fresh weight of callus (0.99±0.08 g) followed by leaf explants (0.75±0.63 g) cultured on MS medium supplemented with 1.0 mg/L BAP and 1.0 mg/L 2,4-D. The lowest fresh weight (0.05 ±0.01) was observed in petiole explants cultured on MS medium supplemented with 1.0 mg/L BAP and 1.0 mg/L NAA. Combination of 2.0 mg/L BAP and 1.0 mg/L 2,4-D also produced the

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highest growth index (160.32±29.32) in stem explants. The results clearly indicate that stem explants are the most suitable explants for callus induction with a better morphology and biomass production in *C. vespertilionis.* The same observation was reported in production of callus from stem explants of important medicinal plants such as of *Jatropa curcas* [17], *Talinum triangulare* [18] and *Withania coagulans* [19].

**Table 2.0**: Effects of different combinations and concentrations of BAP, NAA and 2,4-D on percentage of callus production of *C. vespertilionis.*

| Plant Growth Regulators (mg/L) | | | Percentage of response (%) | | |
|---|---|---|---|---|---|
| BAP | NAA | 2,4-D | Petiole | Stem | Leaf |
| 1.0 | 1.0 | - | 100 | 77 | 88 |
| 1.5 | 1.0 | - | 100 | 100 | 77 |
| 2.0 | 1.0 | - | 100 | 100 | 100 |
| 1.0 | - | 1.0 | 100 | 100 | 100 |
| 1.5 | - | 1.0 | 100 | 100 | 100 |
| 2.0 | - | 1.0 | 100 | 100 | 100 |

### *C. Regeneration of multiple shoots*

The regeneration of multiple shoots was significantly affected by the different combinations and concentrations of plant growth regulators. In the present study, stem explants are the more responsive in the production of shoots with 67% of shoot multiplication in MS medium supplemented with 2.0 mg/L BAP and 1.0 mg/L 2,4-D (Table 5) (Fig. 3). The highest number of multiple shoots was recorded in stem explants cultured on the same medium. There was no regeneration of shoots was observed in petiole explants. The results showed that the increasing of concentration of BAP will increase the production of shoots. It has been well reported that regeneration of multiple shoots are highly affected by optimum concentrations of cytokinins [18]-[19].

## CONCLUSION

This study is the first report related to *in vitro* germination and propagation of *C. vespertilionis* with different pretreatments and types of explants, respectively. The overall results indicated that the medium composition significantly affected the formation of callus and regeneration of shoots in *C. vespertilionis.* This study also provides an idea for efficient production of multiple shoots from *in vitro* germinated seedlings. The results may provide important information in future works for mass production of this medicinal plant for commercial purposes.

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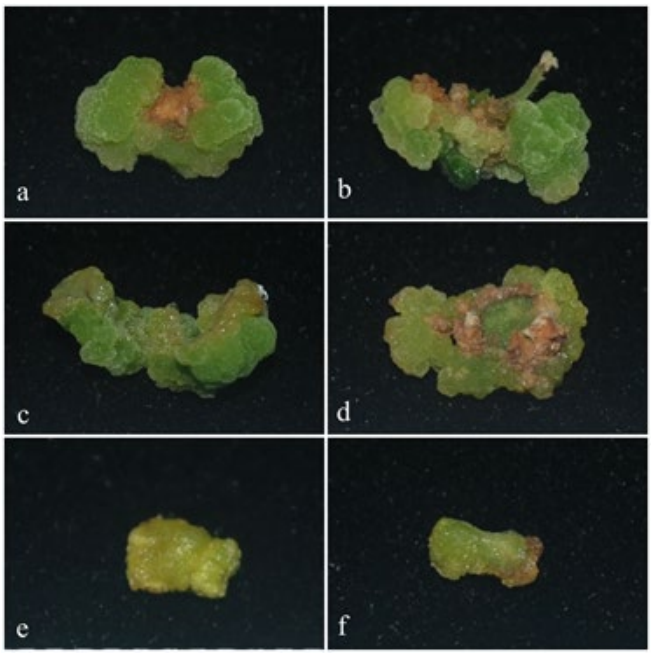


**Fig. 2**: Callus induction in different explants of *C. vespertilionis* cultured on MS medium augmented with combination of BAP and 2,4-D after six weeks of culture. (a) and (b) Green and friable callus formed in stem explants. (c) Green and friable callus formed in leaf explants (d) The leaf explants turned brown with green and friable callus. (e) and (f) Less production of callus in petiole explants.

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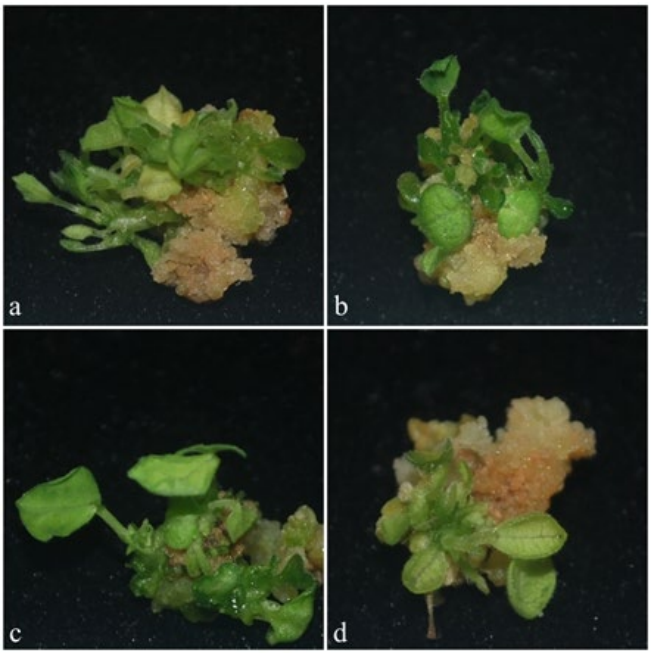


**Fig. 3**: Regeneration of multiple shoots in stem explants of *C. vespertilionis* after eight weeks of culture. (a), (b) and (c) Shoot multiplication on MS medium augmented with 2.0 mg/L BAP and 1/0 mg/L 2,4-D. (d) Shoot multiplication on MS medium augmented with 1.0 mg/L BAP and 1.0 mg/L NAA.

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**Table 3:** Effects of different concentrations and combinations of BAP, NAA and 2,4-D on morphology and colour of callus of *C. vespertilionis*

| Plant Growth Regulators (mg/L) | | | Morphology of Callus | | | Colour of Callus | | |
|---|---|---|---|---|---|---|---|---|
| BAP | NAA | 2,4-D | Petiole | Stem | Leaf | Petiole | Stem | Leaf |
| 1.0 | 1.0 | - | C | F | F | G | G | G |
| 1.5 | 1.0 | - | C, F | C, F | C | G | G | G |
| 2.0 | 1.0 | - | C | C, F | F | G | G | G |
| 1.0 | - | 1.0 | C | C, F | F, C | YG | YG | G |
| 1.5 | - | 1.0 | C | C | F, C | G | YG | BG |
| 2.0 | - | 1.0 | C | F | F | G | YG | BG |

C-compact, F-friable, G-green, YG-yellowish green, BG-brownish green.

**Table 4:** Effects of different concentrations and combinations of BAP, NAA and 2,4-D on fresh biomass and growth index of callus of *C. vespertilionis.*

| Plant Growth Regulators (mg/L) | | | Fresh Weight of Callus (g) | | | Growth Index of Callus (GI) | | |
|---|---|---|---|---|---|---|---|---|
| BAP | NAA | 2,4-D | Petiole | Stem | Leaf | Petiole | Stem | Leaf |
| 1.0 | 1.0 | - | 0.05 ±0.01a | 0.20±0.01ab | 0.16±0.03b | 10.09±4.23a | 25.73±1.16a | 15.42±1.18a |
| 1.5 | 1.0 | - | 0.06±0.01a | 0.59±0.06c | 0.18±0.07b | 11.40±1.80a | 72.38±3.17ab | 32.66±4.22b |
| 2.0 | 1.0 | - | 0.03±0.00a | 0.10±0.01a | 0.02±0.00a | 13.23±0.79a | 18.58±1.46a | 17.48±1.34a |
| 1.0 | - | 1.0 | 0.24±0.10b | 0.43±0.15bc | 0.75±0.63d | 32.68±17.74b | 107.81±62.72ab | 75.48±2.19d |
| 1.5 | - | 1.0 | 0.15±0.03ab | 0.43±0.54bc | 0.42±0.17c | 17.99±2.21a | 130.87±11.98b | 87.12±2.91e |
| 2.0 | - | 1.0 | 0.41±0.02c | 0.99±0.08d | 0.26±0.16b | 59.10±3.73c | 160.32±29.32b | 66.51±3.47c |

Mean values followed by the same letter were not significantly differently ($p < 0.05$).

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**Table 5:** Effects of different concentrations and combinations of BAP, NAA and 2,4-D on shoot multiplication of *C. vespertilionis.*

| Plant Growth Regulators (mg/L) | | | Percentage of Shoot Multiplication (%) | | | Number of Shoot | | |
|---|---|---|---|---|---|---|---|---|
| BAP | NAA | 2,4-D | Petiole | Stem | Leaf | Petiole | Stem | Leaf |
| 1.0 | 1.0 | - | 0 | 50 | 0 | 0 | 3 | 0 |
| 1.5 | 1.0 | - | 0 | 0 | 0 | 0 | 0 | 0 |
| 2.0 | 1.0 | - | 0 | 33 | 0 | 0 | 1 | 0 |
| 1.0 | - | 1.0 | 0 | 33 | 33 | 0 | 1 | 1 |
| 1.5 | - | 1.0 | 0 | 0 | 0 | 0 | 0 | 0 |
| 2.0 | - | 1.0 | 0 | 67 | 0 | 0 | 5 | 0 |