In vitro Embryo Germination and Callus Induction of *Cynometra cauliflora*, an Underutilized Medicinal Plant

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ABSTRACT: *Cynometra cauliflora* or known as 'Nam-Nam' is an underutilized medicinal plant of Malaysia. In this study, embryo from matured seed of *C. cauliflora* was cultured on the Murashige & Skoog medium containing 0.5 µM BAP. Embryo germination achieved up to 100% and produced an average of 8.8±2.0 shoot buds per explant and 1.7±1.5 leaves after eight weeks. Stem and shoot tips explants from in vitro plantlets were further treated with 5, 10 and 15µM Thidiazuron (TDZ) for regeneration capacity. Stem explant responded 100% to callus induction after two weeks of culture on medium containing 10 µM and 15µM TDZ. Meanwhile, shoot tip explant recorded only 12.11±0.54% to callus induction after 6 weeks of culture on medium containing 5µM TDZ. Stem explant remain at calli stages until the end of study, while shoot tips undergo shoot regeneration by inducing up to 54.45±9.27% shoots production with an average of 3.00±1.00 shoots per explant. Browning phenomenon was severely observed on the culture throughout this study. Therefore, the addition of activated charcoal to the culture medium will be beneficial to eliminate phenolics accumulation.

KEYWORDS: Callus; Embryo explants; Micropropagation; Nam-nam; Plant growth regulator

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INTRODUCTION

Cynometra cauliflora L. or commonly known as 'Nam-Nam' among native Malaysian is a medicinal plant belongs to the Fabaceae family. Other vernacular names are Katong-Katong (Sabah) and Buah Katak Puru (Peninsular Malaysia) (Lim, 2012). The fruit which are kidney-shape pod, greenish yellow to brown, with a rough and wrinkled surface can be consumed as fruit salad (*ulam*), pickles, or used as a condiment based ponded chili (Burkill, 1966; Lim, 2012). The leaf was reportedly rich with biological activities such as anticholinesterase, antityrosinase, and α-glucosidase inhibitory activities (Ado *et al*. 2015). Phytochemicals such as tannin, saponins and flavonoid are presence in several parts including young leaf, matured leaf, stem and barkwhich contributed for antioxidant potential of the plant (Aziz & Iqbal, 2013). Meanwhile, extract of the whole fruit showed cytotoxicity towards human promyelocytic leukemia HL60 –Cells (Tajudin *et al*., 2012)

Despite of its richness in bioactivities, *C. cauliflora* has been classified among the 36 species of threatened plants due to high rates of seed dormancy. It has slow growth rate and takes up to 20 years to reach a height of two meters thus make it rare and difficult to find (Jayasuriya *et al*., 2012). Moreover, the plants producing abundant flowers but the fruit development was only approximately less than 7%. This may be influenced by several factors such as pollination vectors, pests as well as natural factor (Purwantoro *et al*., 2010). Hence, *in vitro* propagation technique that allows production of large number of plants from small pieces of plant stock in relatively short

periods of time (Akin-Idowu *et al*., 2009) may useful to overcome this problem. Manipulation of factors such as plant growth regulators and selection of explant play an important role in regeneration potential of particular plant species. The plant hormones auxin and cytokinin are critical for plant regeneration in tissue culture, with cytokinin playing an instrumental role in shoot organogenesis (Hills & Schaler, 2013). Protocol for *in vitro* regeneration system has been well established in other Fabaceae species such as *Clitoria ternata* (Singh & Tiwari, 2012), *Oxytropis glabra* (He *et al*., 2015) and *Acaciella angustissima* (Herrada *et al*., 2016). To our knowledge, there is no micropropagation protocol has been reported for *C. cauliflora*. Therefore, the objectives of this study are to determine the effect of 6-benzylaminopurine (BAP) on *in vitro* embryo germination and the effect of Thidiazuron (TDZ) on shoot regeneration capacity of *in vitro* stem and shoot tips explant of *C. cauliflora*.

METHODOLOGY

Plant Material and Explant Preparation

The fruit of *C. cauliflora* were collected from RPT Batu Mengkebang, Kuala Krai, Kelantan (Figure 1a). They were cleaned in running tap water and then surface sterilized by immersed in 99.9% (v/v) Clorox added with two drops of Tween 20 for 10 minutes. The fruit were then soaked in 70% (v/v) industrial ethanol for 90 second and finally rinsed three times in sterile distilled water. The sterilized fruit was transferred to a glass petri dish and dried on sterile tissue paper prior to the experiment.

Culture Medium and Condition

MS (Murashige & Skoog, 1962) was used as basal medium supplemented with 3% (w/v) sucrose. Medium was fortified with different plant growth regulators in different experiments. The pH of all media was adjusted to 5.8 using 1 N NaOH or 1 N HCl prior to addition of 0.8% (w/v) agar and then autoclaved at 121 ◦C for 15 min. All cultures were incubated at 24±2 ^{0}C in a culture room and were exposed to 24 h light provided by cool fluorescent light.

Effect of BAP on embryo germination

The fruit flesh was removed under sterile conditions using fine forceps and scalpel. Seeds were cut into halves and embryos were isolated by longitudinal excision of the seed (Figure 1b-e). Embryo was cultured on MS medium supplemented with 0.5 µM BAP and hormone-free medium served as control. After 8 weeks of culture initiation, the effect of BAP was determined by recording the percentage of germination, number of shoot bud, number of leaf as well as shoot and root length.

Effect of TDZ on shoot regeneration from stem and shoot tip explants

Stem and shoot tip excised from 10 weeks-old *in vitro* plantlets were cultured on MS medium fortified with 5, 10 and 15 µM TDZ and hormone-free medium served as control. The effeciency of TDZ on regeneration capacity was determined by recording percentage of explant response, callus or shoot induction, number of shoot after 8 weeks on culture initiation.

Experimental design and statistical analysis

All experiments were carried out in a completely randomized design (CRD) with three replicates per treatment and each replicate contains three explants. Data were subjected to analysis of variance (ANOVA) or *t*-test, and the means value were compared by the Duncan's multiple range test at $p<0.05$ using the SPSS ver. 20 (SPSS Inc., USA).

RESULT AND DISCUSSION

Effect of BAP on Embryo Germination

Aseptic seedling of *C. cauliflora* was successfully established in control and MS medium supplemented with 0.5 µM BAP after 8 weeks of culture (Table 1 and Figure 1f-j). There were no significant differences between media tested. The embryo germinated on the second week of incubation achieved 100% germination in both treatments. It was known that MS basal media are composed of inorganic salts plus a few organic nutrients and vitamins that required for the growth of initial explant. Successful germination of embryo in the basal medium lack of plant growth regulators indicate that the presence of endogenous hormone in embryo explant may sufficient for the growth and development of shoots and roots (Miransari & Smith, 2014). Similar results were also reported for *in vitro* embryo culture of *Taxus baccata* (Tafreshi *et al*., 2011) and *in vitro* seed germination of *Acaciella angustissima* (Herrada *et al*., 2016).

During the second week of incubation, root emerged from the embryo (Figure 1f) followed by shoot initiation after 3 weeks of culture and continuously elongated up to 4 weeks of culture (Figure 1g) in medium supplemented with 0.5 BAP. This result is comparatively better than in control medium (shoot emergence at 4 week) and conventional seed germination (shoot emergence at 6 week) of *C. cauliflora* (Jayasuriya *et al.* 2012). The presence of 0.5 µM BAP in the culture medium accelerated embryo germination and seedling development where seedlings produced 1-2 expanded leaves after 8 weeks of culture (Figure 1(j)). However, for the same period of culture in control medium, seedling development was still at the stage of leaf initiation. Cytokinin was essential to induce shoot bud break and shoot multiplication. BAP for example when added alone in the medium showed the best response for shoot regeneration from decapitated embryo axes of *Clitoria ternata* (Singh & Tiwari, 2012). An average of 7.4-8.8 shoot buds per explants along with 1.0-1.7 leaves per explant was observed on both treatments. However, control medium showed slightly longer root (42.4±4.2 mm) compared to BAP-containing medium (33.8±3.1 mm). The stimulating effect of BAP for seed germination and shoots regeneration has been reported earlier in other plant species such as *Pterocarpus santalinus* (Balaraju *et al*., 2011) and *Oxytropis glabra* (He *et al*., 2015).

Table 1. Effect of BAP on embryo germination of *C. cauliflora* after 8 weeks of culture on MS basal medium supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar, pH 5.8. Culture incubated at 25°C±2°C under 24h light condition

Treatment medium	Percentage of embryo germination (%)	Mean of shoot buds/ explant (Mean±S.D)	Mean of shoot height (mm) (Mean±S.D)	Time for shoot emergence (week)	Mean of number of leaf/explant (Mean±S.D)	Mean of root length (mm) (Mean±S.D)	Time for root emergence
Control	100	7.4±3.7	18.1±3.0	4	1.0±1.0	42.4±4.2	2
0.5µM BAP	100	8.8±2.0	16.8±6.6	3	1.7±1.3	33.8±3.1	2

Figure 1. *In vitro* embryo germination of *Cynometra cauliflora* on MS basal medium supplemented with 0.5µM BAP within 8 weeks of culture.(a) Tree of *C. cauliflora* (b) fruit of *C. cauliflora* (c) Seed of *C. cauliflora* (arrow) after fruit flesh was removed (d) seed cut longitudinal into halves and attached embryo (black circle) (e) embryo culture initiation (f) emergence of root after 2 weeks of culture (g) shoot emergence and elongation (h) initiation of shoot buds (i-j) formation of the first leaf and leaf expanded after 8 weeks of culture

Effect of TDZ on Regeneration Capacity From Stem and Shoot Tip Explant

The stem explants cultured on MS medium supplemented with higher concentrations of TDZ (10-15 µM) resulted in heavy callusing and successfully promotes up to 100% friable callus at the second week of culture (Table 2 and Figure 2d). Similar result has also been reported in *Cassia sophera* (Parveen & Shahzad, 2010). However at lower concentration (5µM), callus induction was delayed up to 4 weeks. For shoot tip, inclusion of TDZ caused a swelling of the explants after 2-3 weeks of culture (Figure 2b). Callus induction was observed at the base of the explants within 6 weeks. After 6 weeks, indirect shoot regeneration from callus was observed on medium supplemented with TDZ (Figure 2d). Shoot was induced in all concentrations of TDZ and the number was reduces with increased concentration from 5-15 µM.

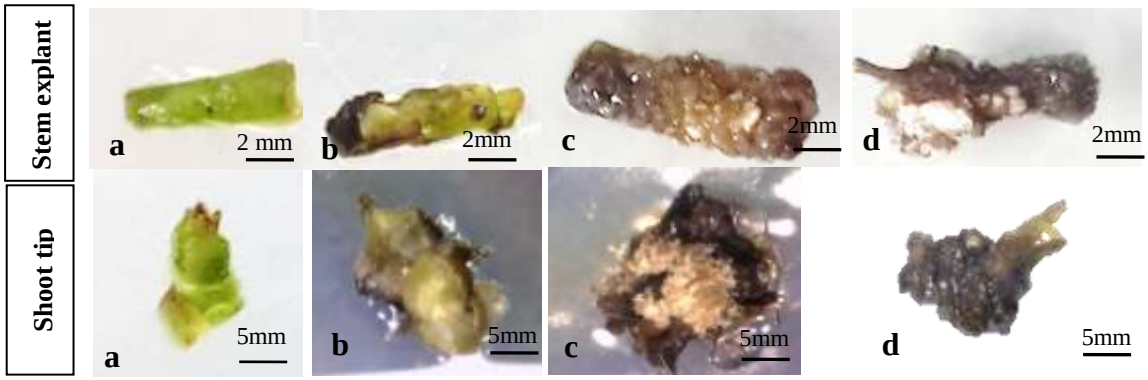

Figure 2. Regeneration capacity of *C. cauliflora in vitro* stem and shoot tip explant on MS basal medium supplemented with TDZ. **Stem explant**: (a) Culture initiation (b) Explant started to swell after two weeks of culture.(c-d) initiation and callus formation after 2-3 weeks of culture. **Shoot tip explant:** (a) Culture initiation (b) Explant started to swell after two weeks of culture. (c) callus initiation at the base of explant (d) shoot regeneration after 6 weeks of culture

Table 2. Effect of TDZ treatment on regeneration capacity of *C. cauliflora* stem and shoot tip explant after 8 weeks of culture supplemented with 3% (w/v) sucrose and 0.8% (w/v) agar.

Explant	TDZ (μM)	Callus induction Percentage (%)	Time for callus induction (week)	Callus Morphology	Percentage of explant produce shoot (%)	Mean number of shoot per explant
Stem	Control	0	-	-	0	0
	5	88.89 ± 9.22^{b}	4	Friable, brownish yellow	0	0
	10	100^{a}	2	Friable, brownish yellow	0	0
	15	100^{a}	2	Friable, brownish yellow	0	0
Shoot tip	Control	0	-	-	33.34 ± 3.35^{b}	0.33 ± 0.00^{d}
	5	12.11 ± 0.54^{c}	6	Friable, brownish yellow	54.45 ± 9.27^{a}	3.00 ± 1.00^{a}
	10	10.21 ± 1.24^{c}	6	Friable, brownish yellow	43.33 ± 3.35^{a}	1.33 ± 0.50^{b}
	15	9.15 ± 0.14^{cd}	6	Friable, brownish yellow	34.44 ± 9.27^{ab}	0.83 ± 0.40^{c}

Mean followed by the same letter in the same column were not significantly different at $p<0.05$ by Duncan's Multiple Range Test.

In comparison with stem, shoot tip showed potential for regeneration by obtaining 34.44% to 54.45% explants to produce shoot. The highest number of shoot per explant (3.00±1.00) was recorded at lower concentration (5 μM TDZ). However, shoot tips took 6 weeks for callus induction and obtained low percentage of callus induction (12.11%). The ability of shoot tips for regeneration was also observed in *Pterocarpus santalinus* (Balaraju *et al.*, 2011). The meristematic ends of the plants like shoot tip have high rates of cell division and either concentrate or produce required growth regulating substances including auxins and cytokinins (Akin-Idowu *et al.*, 2009).

TDZ have both cytokinin and auxin effects (Guo *et al*., 2011) that may resulted to the formation of both callus and shoot in this plant. However, browning phenomenon was observed in both stem and shoot tips explants as well as the induced callus (Figure 2). Wounding the material in order to remove explants and culturing them in potentially stressful environments; often eliciting the production and release of phenolic compounds that resulted to this phenomenon (Jones & Saxena, 2013). It was also may be due to high phenolic content in the plant part as reported earlier by Aziz and Iqbal (2013). Browning of callus in medium supplemented with TDZ was also observed in other Fabaceae species *Uraria picta* (Ahire *et al*., 2011). Phenolic problem in plant tissue culture simply can be overcome by addition of activated carbon. Activated carbon is able to absorbed phenolics and their oxidates thus make them less toxic (Thomas, 2008).

CONCLUSION

In vitro culture of *C. cauliflora* was successfully established from embryo on MS medium supplemented with 0.5 µM BAP. In the presence of TDZ, shoot tips and stem explant showed potential for shoot regeneration and callus induction, respectively. However, the use of antioxidative agent is suggested to overcome browning effect of the culture. The results described in this study provides basis for further improvement of complete plant regeneration system of this plant.

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