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The prospect of micropropagating *Gonystylus bancanus* (Miq.) Kurz, a tropical peat swamp forest timber species through tissue culture technique – Review

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Abstract

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Gonystylus bancanus (Miquel) Kurz is one of the most valuable timber species in tropical peat swamp forest. Its timber is widely used for furniture, decorative cabinets and interior decoration. Due to its higher demand, *G. bancanus* continues to be harvested which leads to its disappearance in the wild. This is worsened by the fact that germination rates in the wild are very poor due to rapid insect and fungal infestation. Even though vegetative propagation via stem cuttings has been successful, proper protocols on tissue culture need to be explored as a viable way of mass production of a large number of planting stock in a relatively shorter time for forest rehabilitation and enrichment planting purposes.

Keywords: leaf segment; nodal stem segment; organogenesis; shoot induction; somatic embryogenesis; sterilization

Gonystylus bancanus (Miquel) Kurz is a medium to fairly large tree, which can reach up to 40 m in height with approximately up to 120 cm DBH (Kartiko 2002). It appears to have irregular flowering and fruiting habits, also referred to as 'supra-annual', when it flowers once in two to three years (Ismail 2009). A question is being raised whether timber harvesting activities in peat swamp forest, even though carried out strictly in a sustainable manner using reduced impact logging techniques, could put pressure on *G. bancanus*, which grows in clumps and any attempt to take out bigger trees could affect the growth and survival of nearby smaller trees.

Tissue culture technique offers a viable alternative to micropropagate *G. bancanus* as it offers mass production of plants in a shorter time (Kumar, Reddy 2011). It involves the use of small pieces of plant tissue (explants) which are cultured in a nutrient medium under sterile conditions to rapidly produce new shoots, using appropriate growing conditions for each explant type, and with the addition of suitable hormones new roots would be initiated. These plantlets can be divided

further, usually at the shoot induction stage and subcultured into a medium with new plant growth hormone to produce large numbers of new plantlets. The new plantlets could then be acclimatized to soil conditions and grown in the nursery. This paper highlights and discusses the importance of tissue culture study, research achievements and endeavours as well as challenges hindering the practical application of tissue culture in some earlier works on *G. bancanus* and of some other important tropical timber species as a prospect of further improving tissue culture protocols for *G. bancanus*.

Conventional propagation of *G. bancanus*

Since the implementation of sustainable forest management practices in Peninsular Malaysia to manage permanent forest reserve, reforestation activities of logged-over forest and enrichment planting depends very much on seedlings collected from the wilds, mostly from the surrounding areas so as to maintain species composition in the area, even though natural grown seeds were reported to grow fairly slowly (Shamsudin 1996). Even though vegetative propagation was seen as an alternative as it saves time and can produce uniform planting stock continuously throughout the year from selected parental stock, this technique has its own disadvantages such as the homogenization of the planting material which could increase vulnerability of planting material to both existing and novel pests and pathogens, as reported in some crop species (Zhu et al. 2000). Contrary to the case of the neighbouring country like Indonesia where the majority of enrichment planting uses planting materials produced from vegetative stem cuttings (Weinland 1998), vegetative propagation in Malaysia has remained largely experimental (Kettle 2010). No forest rehabilitation activities such as replanting on the open areas, degraded and encroached forest areas.

Vegetative propagation techniques through stem cuttings were reported to be possible for *G. bancanus* with various degree of success as reported by Mohamad Lokmal et al. (1993), Hendromono (1999) and Nor Aini et al. (2010). The techniques were also used to propagate other commercial tropical timber species such as *Shorea parvifolia* Dyer and *Shorea acuminata* Dyer (Nor Aini, Ling 1993), *Shorea leprosula* Miquel (Aminah et al. 1997), *Shorea macrophylla* (de Vriese) Ashton (Yen-Nyuk 1985) and *Dryobalanops lanceolata* Burck (Moura-Costa, Lundoh 1994), *Hopea odorata* Roxburgh (Aminah 1991), *Madhuca motleyana* (de Vriese) J.F. Macbride, *Anisoptera marginata* Korthals, *Stemonurus secundiflorus* Blume, *Calophyllum* spp., *Durio carinatus* Masters, *Santiria* spp., *Pometia pinnata* J.R. Forster & G. Forster and *Shorea platycarpa* Heim (Ismail, Shamsudin 2003).

Micropropagation of some tropical forest tree species via tissue culture

Micropropagation of forest tree species especially those with commercial timber values via tissue culture was said to be a viable method for producing plants for reforestation, conventional breeding and mass propagation (Ortiz et al. 2000). If compared with the rapid progress of the breeding programmes for two popular exotic genera such as *Acacia* Miller and *Eucalyptus* L'Héritier de Brutelle, breeding via tissue culture of indigenous tropical timber species in particular the peat swamp forest species such as *G. bancanus* is well behind. This is partly due to the fact that there is no plantation area of the indigenous species as yet, in contrast to the exotic species, which have been planted on over millions of hectares and it is by far mainly for enrichment planting and forest rehabilitations purposes.

Earlier summary on tissue culture of some notable tropical timber species has been compiled and detailed out by Vaario (1996), where it was pointed out that the earliest tissue culture works on dipterocarps were reported by Smits and Struycken (1983) in their work on three *Shorea* Roxburgh ex C.F. Gaertner species vis *Shorea curtisii* Dyer ex King and *Shorea obtusa* Wallich ex Blume as well as *Dipterocarpus grandiflorus* Blanco. Since then, few other works have been carried out on various dipterocarp species such as *H. odorata* (Scott et al. 1987), *Shorea robusta* Roth (Jain, Chaturvedi 1991), *Dipterocarpus alatus* Roxburgh and *Dipterocarpus intricatus* Dyer (Linington 1991), *S. leprosula* (Vaario et al. 1993), and *Vatica diospyroides* Symington (Srisawat, Jongkraijak 2013) with various degree of success. It is interesting to note that some of the species such as *D. alatus*, *D. intricatus* (Linington 1991) and *V. diospyroides* (Srisawat, Jongkraijak 2013) are actually yet to show promising organogenic response from meristematic explant sources such as the shoot tips and nodal segment as compared to embryonic explants, even though the meristematic tissue used as explant was considered an area of active plant growth where undifferentiated cells divide and

form new, specialized cells. This evoked perception that tropical forest trees were indeed recalcitrant in nature and any attempt to manipulate their growth especially *in vitro* requires modification of protocols such as pre-treatment during sterilization and addition of a higher concentration of plant growth regulators to stimulate cell differentiation.

Micropropagation of *G. bancanus* and some other peat swamp forest species via tissue culture

The first peat swamp forest timber species to be micropropagated through tissue cultures was *Shorea albida* Symington, an important peat swamp forest timber species in the island of Borneo. The study reported swelling and callusing of shoot tip explants but no further development was attempted (Ishii, Mohsin 1994). As for *G. bancanus*, the first attempt was reported by Rosilah et al. (2006) when they successfully initiated callus from stem explants. Since then few other studies have reported successful direct organogenesis using shoot tip explants (Yelnititis, Komar 2011; Chieng et al. 2014a) and callus induction (Yelnititis 2012; Chieng et al. 2014b; Yelnititis 2014; Putri 2015) (Table 1). The most commonly used medium in the work with this species was full-strength Murashige and Skoog (MS) medium with other media preferences such as Gamborg's B5 and Woody Plant Medium (WPM) were reported with various degree of success. These media were supplemented with various types of plant growth regulators depending on the desired type's development. Plant growth regulators such as 6-benzylaminopurine (BAP) were the most commonly used cytokinins for shoot proliferation while naphthaleneacetic acid (NAA) and 2,4-dichlorophenoxyacetic acid were used for callus induction and eventually somatic embryogenesis.

Direct organogenesis

Organogenesis can be described as a process by which a cell or group of cells differentiate to form organs. Tomar and Gupta (1988) defined organogenesis as the reflection of the intrinsic genetic constitution of a taxon, of which equally refers to the formation of roots or shoots. Organogenesis is commonly induced by manipulation of exogenous phytohormone levels and occurs directly either from explant tissue or through callus. Practically any plant part can be used for organogenesis, but the most commonly used for woody species are shoot tip, nodal segment, ovular tissue, inflorescence section, cotyledons and embryos (Thorpe 1994). These explants were also considered direct pathway for plant regeneration and usually the first to be attempted with, of which were reported to be successful for *G. bancanus* to produce shoot both from shoot tip (Chieng et al. 2014a; Putri 2015) and nodal segment explant (Yelnititis, Komar 2011; Chieng et al. 2014b). The explant sources used in all the studies were obtained both from aseptically germinated and wild seedlings. Similar explant types (shoot tip and nodal stem) were reported to be used in other earlier work on tropical timber species such as *Shorea johorensis* Foxworthy (Vaario 1996) and *S. leprosula* (Vaario et al. 1993) where they were reported to be producing shoots successfully.

It is interesting to note that except for the work by Chieng et al. (2014a), who used WPM, previ-

Table 1. Applications of *in vitro* cultures for *Gonystylus bancanus* (Miquel) Kurz and other peat swamp forest species

Species (local name)	Medium	Plant growth regulators	Explants	Morphogenic response	Reference
Shorea albida Symington (Alan)	Gamborg's	IBA, BAP + NAA	shoot tip nodal stem	swelling callus	Ishii and Mohsin (1994)
G. bancanus (Ramin melawis)	MS	BA, kinetin, TDZ	nodal stem	shoots	Yelnititis and Komar (2011)
	MS	2,4-D + TDZ	leaf	callus	Yelnititis (2012)
	MS	BAP	shoot tip nodal stem	shoots	Chieng et al. (2014a)
	MS	NAA	lamina	callus	Chieng et al. (2014b)
	WPM	2,4-D			
	MS	2,4-D	leaf	callus	Yelnititis (2014)
	MS	BAP, NAA, kinetin	auxiliary shoot	shoots	Putri (2015)

MS – Murashige and Skoog, WPM – Woody Plant Medium, IBA – indole-3-butyric acid, BAP – 6-benzylaminopurine, NAA – naphthaleneacetic acid, BA – 6-benzyladenine, TDZ – thidiazuron, 2,4-D – 2,4-dichlorophenoxy acetic acid

ous work on *G. bancanus* mainly used MS medium (Murashige, Skoog 1962) for either shoot initiation, callus induction, somatic embryogenesis or rooting process. The reasons for these preferences were not pointed out but it is well-known that MS medium, which has a very high concentration of nitrate, potassium and ammonia as well as inorganic nutrients, was preferred over other commonly used media for woody species such as WPM and Gamborg's B5 medium. Earlier tissue culture works on most tropical timber species such as *H. odorata* (Scott et al. 1995), *Shorea roxburghii* G. Don (Nakamura 1996), *V. diospyroides* and *S. leprosula* (Vaario et al. 1993) were also reported to use MS medium.

Plant growth regulators play vital roles in the growth and differentiation of plant tissues. Different plant growth regulators have different effects and they vary with the type and quantity to be applied. A higher level of cytokinin to auxin ratio promotes shoot formation, while a higher auxin to cytokinin ratio favours root differentiation (Kataria et al. 2013). It is not clear whether the application of cytokinins such as BAP and kinetin (6-furfurylaminopurine) could have favourable effects on shoot proliferation of *G. bancanus* but in other tropical forest species such as *H. odorata* (Scott et al. 1995) they were reported to have a positive influence on shoot initiation when applied singly at a lower concentration (0–5.0 $mg \cdot l^{-1}$). On the other hand, combinations with other cytokinins such as kinetin (Chieng et al. 2014b) or auxins such as NAA (Putri 2015) were reported to be successful in initiating shoot proliferation in *G. bancanus*.

Somatic embryogenesis

Plant regeneration from somatic embryogenesis has been considered to be important and gained attention in forestry biotechnology for two reasons. Firstly, the system offers the capability to produce unlimited numbers of somatic embryo derived propagules and artificial/synthetic seeds. Secondly, the embryogenic culture system could also be used efficiently for genetic transformation studies (Giri et. al. 2004). However, the most notable aspect of somatic embryogenesis is that it has a single cell origin as opposed to other multicellular events such as organogenesis and micropropagation. Somatic embryogenesis from non-gametic cells can produce a bipolar embryo with similar developmental stages that occur in the normal zygotic embryo.

The progress and the advancement of somatic embryogenesis in tropical forest timber species in general can be considered as problematic depending on a lot of factors such as explant types and age, media and plant growth hormone. Earlier attempts to initiate somatic embryogenesis on *G. bancanus* by Yelnititis (2012) and Chieng et al. (2014b) have reported successful production of somatic embryos directly or through embryogenic callus phase. The callus produced was friable and yellowish in colour. However, no further plantlet development was reported in both studies. A study on *V. diospyroides* also reported unsuccessful regeneration of plantlets from embryogenic calli once they were transferred onto regeneration medium (Srisawat, Jongkraijak 2013). These warrant further refinement in the tissue culture protocols employed in those studies especially in understanding the requirements for efficient regeneration of somatic embryos, which sounds impossible, without any callus pathway and also improve the low conversion frequencies in embryogenic calli.

Basically, any plant parts can be used to induce somatic embryogenesis in most tree species and most have been tried with various degree of success on immature zygotic embryos of *Acacia mangium* Willdenow (Xie, Hong 2001) and *Acacia nilotica* (Linnaeus) Willdenow ex Delile (Garg et al. 1996), cotyledons of *Acacia catechu* P.S.H. Hurter & Mabb (Das et al. 1996) and leaf explant of *Acacia sinuata* (Loureiro) Merrill (Vengadesan et al. 2002) while *Eucalyptus globulus* Labill was reported to produce somatic embryos directly from cotyledons (Nugent et al. 2001), leaf (Corredoira et al. 2015) and zygotic embryo explants (Pinto et al. 2002). However, the explants must be from juvenile sources when they are having highly differentiated cells that are capable of undergoing embryogenesis. These immature plant parts, also referred to as meristems, are where cells proliferate and partition into layers that eventually differentiate to form various tissues and organs of the plant like in organogenesis.

MS and WPM media were reported to be equally successful in callus induction and regeneration procedures in most woody species. For specific purposes such as maturation and germination of the somatic embryos, MS basal medium or half-strength MS medium were also widely used. Addition of other media components such as casein hydrolysate and glutamine was reported to improve somatic embryogenesis in *Eucalyptus citriodora* Hook (Muralidharan et al. 1989), however, it did not improve somatic embryo production with

a higher number of abnormal somatic embryos in *E. globulus* Labill (Pinto et al. 2002). In another study on *Quercus rubra* Linnaeus (red oak), Sánchez et al. (2003) reported that sucrose was used as a carbon and energy source, as it enhances somatic embryo maturation by causing osmotic stress when supplied at higher concentrations. Accumulation of food storage, such as sucrose usually converts translucent embryos into white-opaque embryos as reported by Cerezo et al. (2011) and Martínez et al. (2014). Earlier attempt to initiate somatic embryogenesis in *G. bancanus* reported a rather interesting outcome as a higher concentration (40.0 mg·l^{-1}) of NAA was used with success. This is in contrast to most of the work with *A. catechu* (Das et al. 1996) where a lower concentration of NAA (2–3.0 mg·l^{-1}) was applied. This might be due to the recalcitrant nature of *G. bancanus* tissues to somatic embryogenesis of which was also reported for gymnosperms like coconut (*Cocos nucifera* Linnaeus) (Verdeil et al. 1994).

Therefore, it is difficult to point out what is the best level of plant growth regulator to be recommended as different plant types respond differently in different conditions.

Contamination problem

Contamination presents a major challenge in tissue culture work of *G. bancanus* (Chieng et al. 2014a) and other peat swamp forest timber species such as *S. albida* (Ishii, Mohsin 1994) as a higher contamination rate was reported. According to Leifert and Cassells (2001), contaminants in tissue cultures vary in a wide range of microorganisms including filamentous fungi, yeasts, bacteria, viruses, viroids, mites and even thrips. While the fungus may arrive from the explant or airborne, contamination by bacteria was believed to originate from endogenous bacteria that escape initial disinfection or by microorganisms introduced during tissue culture manipulations such as subculture to fresh media. Both types of contaminants may survive in the plant material for several subculture cycles for overextended periods of time without expressing symptoms in the tissue or visible signs in the medium. Generally, the sterilization technique involved pretreatment using ethanol (concentration between 70 and 95%) followed by washing with various concentrations of sodium hypochlorite (commercial Clorox) for a certain period of time and has been employed with various levels of success to some tropical species. On the other hand, Badoni and Chauhan (2010) reported that sodium hypochlorite was a better sterilant than calcium hypochlorite due to bleaching effects of the latter and hence it has been extensively used for studies on plants such as potatoes that have direct contact with soils. This technique was employed by studies on various other tropical forest trees such as *Azadirachta excelsa* (Jack) Jacobs (Jainol 1997), *Canarium odontophyllum* Miquel (Chai et. al. 2010), *S. parvifolia* (Aziah et al. 2013). Jainol (1997) reported that washing with 15% sodium hypochlorite was effective in reducing a contamination rate up to 75% in her study with nodal stem segments of *A. excelsa* while Chai et. al. (2010) reported that the sterilization technique using 70% ethanol for 60 s and 25% sodium hypochlorite added with Tween 20 for 15 min can reduce a contamination rate up to 33% in their study on *C. odontophyllum*. Aziah et al. (2013) reported washing with 20% (v/v) sodium hypochlorite nodal segments excised from nursery-grown seedlings of *S. parvifolia* for 18 min was effective in reducing a contamination rate to only 39%. In studies involving tropical peat swamp species such as *G. bancanus* (Chieng et al. 2014a) and *S. albida* (Ishii, Mohsin 1994), sterilization techniques of submerging explants in 75% ethanol for 1 min followed by sterilization with 0.3 mol mercuric chloride for 5 min failed to eliminate fungus contaminations. It is interesting to note that, even though some experiments may give comparatively highly clean culture when tested using a particular technique, these results are not usually replicable on other tissue culture work and so the expected results are usually elusive (Barrett, Cassells 1994). In many cases anti-microbial treatments only inhibit contaminants and low levels of contamination persist even though some researchers incorporated the use of antibiotics and fungicides to overcome the contamination problem (Leifert, Cassells 2001). Another problem that affects tissue culture work of most tropical tree species is browning. It occurs as a result of the exudation of phenolic compounds. This problem was reported for *Acacia* species such as *A. catechu* (Das et al. 1996) and *A. sinuata* (Vengadesan et al. 2002). Some species such as *E. globulus* were reported to release more phenolic compounds as the level of sucrose in the media was increased. The accumulations of phenolic compounds were believed to influence the production of somatic embryos in this species (Pinto et al. 2002). Activated charcoal has been widely used to control and prevent browning. Srisawat and Jongkraijak (2013) reported that the incorporation of activated charcoal was not only effective in absorbing harmful compounds and

exudates but also able to suppress the exudation of phenolic compounds in *V. diospyroides* culture.

CONCLUSIONS

The use of *in vitro* technique for the propagation of *G. bancanus* was reported to be possible with particular attention to be given to the fine tuning of most effective sterilization techniques as higher contamination rates were reported in all earlier studies especially if field grown materials were to be used as explant sources. In terms of media preference, MS basal medium was found to be the most effective when supplemented with various concentrations of BAP for shoot induction and NAA for rooting and initiation of somatic embryogenic calli. It is also worth noting that more efforts should be placed on the establishment of field trials of the micropropagules produced *in vitro*.

The appropriate *in vitro* technique for *G. bancanus,* as well as for other tropical timber tree species needs further refinement as this technique presents many challenges in production costs, labour required to produce the desired planting material, and potential losses suffered during acclimatization of plants to the field. Many *in vitro* studies involving tropical tree species have not been optimized and implemented in the field due to the hesitation on the part of the government to use the technique fully in any forest rehabilitation program. Conservation through *in vitro* propagation technologies, if performed cautiously, will have a global economic and ecological impact on sustaining tropical forest tree biodiversity.

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