Induction of Organogenesis and Somatic Embryogenesis of *Gonystylus bancanus* (Miq.) Kurz (Ramin) In Sarawak

Linna M.N. Chieng, T.Y. Chen,
S.L. Sim and Doreen K.S. Goh

ITTO-CITES Activity:

***In Vitro* Propagation of *Gonystylus bancanus* (Miq.) Kurz (Ramin) in Sarawak**

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All enquiries should be forwarded to*:*

Chief Executive Officer
SARAWAK FORESTRY Corporation
Lot 218, KCLD, Jalan Tapang, Kota Sentosa
93250 Kuching, Sarawak, Malaysia
Tel: +6082-610088 Fax: 6082-610099
Website: www.sarawakforestry.com
Email : info@sarawakforestry.com

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Contents

List of Figures

List of Tables

List of Abbreviations

2,4-D	2,4-Dichlorophenoxyacetic acid
BAP	6-benzyl amino purine
GA	Gibberellic acid
$HgCL_2$	Mercuric chloride
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	Napthlene acectic acid
PGRs	Plant growth regulators
TDZ	Thidiazuron
WPM	Woody plant medium

Acknowledgements

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Summary

Organogenesis and somatic embryogenesis are the two alternative pathways in plant tissue culture techniques for propagation in addition to direct shoot induction. The present study was carried out to establish protocols for the *in vitro* regeneration of Ramin in Sarawak with or without an intervening callus phase through organogenesis and somatic embryogenesis. New leaves that emerged from the bent sapling in the field were used as source of lamina explants. Leaf samples were sprayed with 0.2% a.i. Mancozeb (fungicide) during collection. Lamina explants were surface-sterilized with Mercuric chloride ($HgCl_2$) at 0.2% for 10 minutes. Optimum basal medium for direct organogenesis is modified Murashige and Skoog (MS) basal medium, while for somatic embryogenesis, the optimum basal medium is Woody Plant Medium (WPM). For both direct and indirect organogenesis, the use of cytokinins alone or in combination with auxins at different concentrations for shoot induction failed to generate positive results. To date, there was no sign of bud or shoot growth detected on the calli obtained. On the other hand, for somatic embryogenesis, NAA was effective in somatic embryos induction. NAA at high concentrations produced yellowish-white compact calli. Somatic embryos formation was recorded at 30, 35 and 40 mg/L NAA respectively.

I. Introduction

The genus *Gonystylus* consists of 31 species of which *Gonystylus bancanus* (Ramin), is the most valuable a light hardwood species growing in the peat swamp forest. It is also harvested for the making of furniture and cabinet decoration (Ng & Shamsudin, 2001). As a result of the high demand, Ramin has been heavily exploited in its native countries. In Sarawak (Malaysia), the Ramin production fell from 521,000 m^3in 1990 to 67,000 m^3 in 2000 (CITES 2004).

According to the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES), Indonesia has included the genus *Gonystylus* in Appendix III in 2001 and up listed it to Appendix II in 2005 (Chen, 2007). Conventional propagation of Ramin is normally through seeds. Ramin propagation using seeds is difficult as flowering is irregular and seeds are recalcitrant (Hendromono, 1999). Ramin seeds are usually damaged by squirrels (Ismail *et al*., 2011) and suffered from insects or fungal attack (Shamsudin, 1996). Therefore, other means of propagation have been explored. This included vegetation propagation through cuttings (Ismail & Shamsudin, 2003; Mohamad Lokmal *et al*., 1992) and tissue culture techniques (Yelnititis & Komar, 2011; Rosilah *et al*. 2006; Shamsudin & Aziah, 1992 cited in Khali Aziz Hamzah *et al*. 2010).

In the use of plant tissue culture technique for propagation, organogenesis and somatic embryogenesis are the two alternative pathways in addition to direct shoot culture. Organogenesis involves inducing the vegetative tissue to form organs (shoot or root) which eventually develop into a complete plantlet (small but whole plant), while, somatic embryogenesis is to induce a piece of somatic (vegetative) tissue to develop an embryogenic callus, leading to the formation of a somatic embryo which germinates into a

complete plantlet. The original plant part used to initiate the culture is known as explant. Through the manipulation of plant growth regulators and culture conditions, the explant may develop shoot and root directly. This is called the direct organogenesis. If the formation of shoot and root need to go through a callus phase then it is called the indirect organogenesis. Plant growth regulators (PGRs), usually the cytokinins i.e. 6-benzylaminopurine (BAP) alone in different concentrations or in combination with an auxin for example, Naphtalene Acetic Acid (NAA) or 2,4-Dichlorophenoxyacetic acid (2,4-D) also at different concentrations are used in the induction of direct and indirect organogenesis. Similarly, in somatic embryogenesis, embryos can be developed directly from the explant (direct somatic embryogenesis) or via a callus phase (indirect somatic embryogenesis). In the induction of somatic embryogenesis, direct or indirect, auxins are used at the initial stage and abscissic acid may be needed at a later stage. To date, there is no report on the organogenesis of Ramin. The first attempt on somatic embryogenesis of Ramin was by Yelnititis & Komar (2010) who reported that embryogenic callus was obtained from leaf explants cultured on 7.0 mg/L 2,4-D combined with 1.5 mg/L Biotin incorporated in a modified MS medium. However, somatic embryos have yet to be generated.

The objective of this study is to develop protocols for the *in vitro* regeneration of *Gonystylus bancanus* (Ramin) in Sarawak with or without an intervening callus phase through organogenesis and somatic embryogenesis.

II. Plant Materials

The source of materials used in this study was the new leaves which have emerged from bent wildings in the field (Lingga, Sri Aman). The newly emerged young leaves provided cleaner explants for culture initiation. According to the age of leaves, they were divided into young (not yet unfold) and recently matured leaves (Figure 1). During the samples collection, the leaves were sprayed with 0.2% a.i. Mancozeb after excising from the wildings before bringing them back to the laboratory.

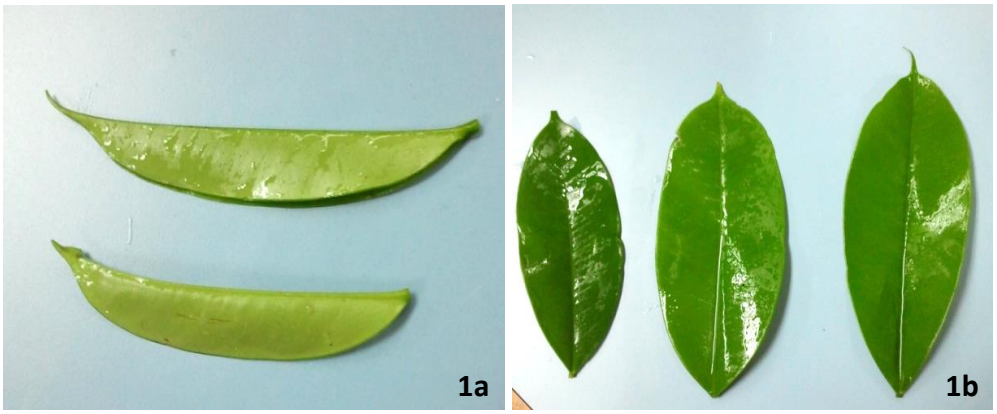

Figure 1 (a) The young and (b) recently matured leaves used as source of explants

III. Surface Sterilization

The leaves were washed gently under running tap water to remove the residue of Mancozeb attached on the surface. After that, the leaves were left under the running tap water for half an hour, air dried and sprayed with 75% Ethanol then air dried again in the laminar flow cabinet. Surface sterilization was carried out according to the best surface sterilization regime developed i.e. submerging the leaves in 0.2% Mercuric Chloride ($HgCl_2$) for 10 minutes with agitation. Subsequently, the sterilant was decanted and the leaves were rinsed five times with sterilized-RO water. Only the lamina was used as the explant. The petioles were discarded. The part of the lamina which was damaged by the sterilant was trimmed off to provide a fresh wound before culturing on the culture medium.

IV. **Direct Organogenesis**

The axenic explants after the surface sterilization process were cultured on the plant regeneration media consisting of 2,4-Dichlorophenoxyacetic (2,4-D) and 6-benzyl amino purine (BAP). According to Dhabhai & Batra (2010), the combination of 2,4-D and BAP in the medium proved effective in callus induction and shoot proliferation of *Acacia nilotica*. Thus, the combination of 2,4-D at 0.1 mg/L with BAP at 1.0, 2.0 or 4.0 mg/L respectively was incorporated into two different basal media i.e. Modified MS (Murashige and Skoog, 1962) and Woody Plant Medium (WPM) (Lloyd and McCown, 1981). The effects of the combinations of 2,4-D and BAP in two different basal media on organogenesis were studied. Callus formation was detected after two weeks of culture initiation. The calli developed in modified MS medium were green, globular and compact in structure (Figure 2a & 2b), whereas calli formed in WPM medium were not green (Figure 3a & 3b) after two months of culture. The green globular calli were believed to have potential in the regeneration of bud or shoot. Arumugam *et al*. (2009) reported the regeneration of *Acacia confusa* shoot bud from compact greenish nodular calli. Therefore, it appeared that the morphology of the callus varied with the type of media used. With this, modified MS medium was chosen for the induction of organogenesis.

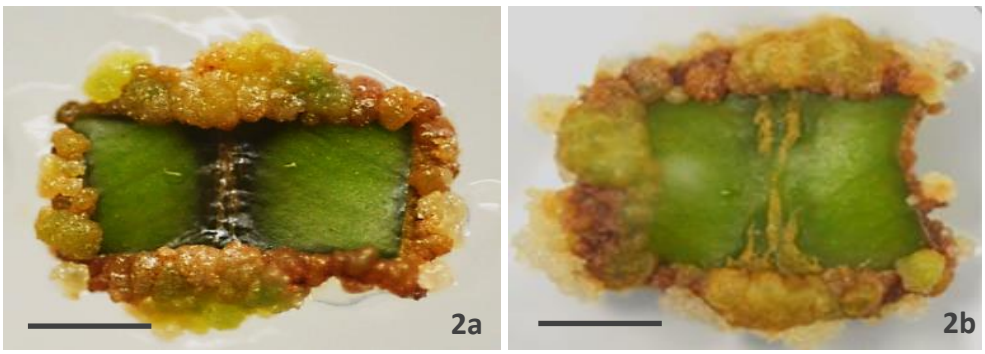

Figure 2 Green, globular structures detected on lamina explants cultured on modified MS medium supplemented with 0.1 mg/L 2,4-D in combination with BAP at (a) 1.0 mg/L 2.0 mg/L respectively (Bar=1cm)

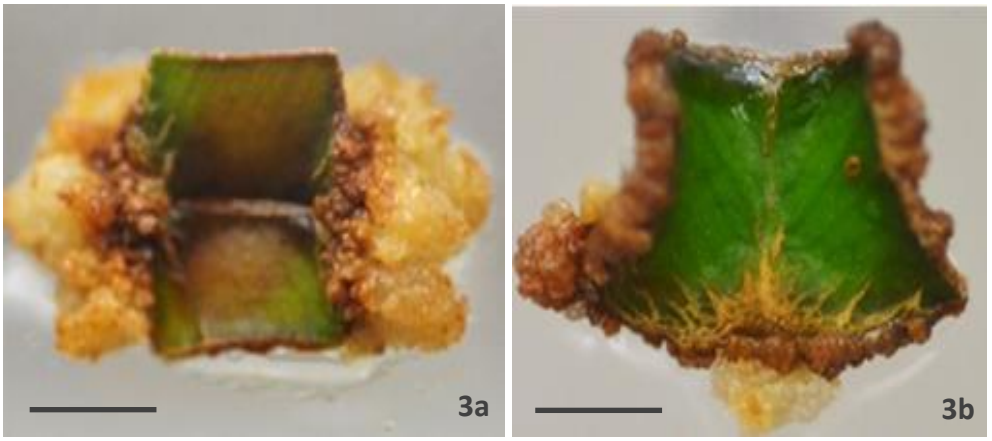

Figure 3 Lamina explants cultured on WPM medium supplemented with 0.1 mg/L 2,4-D incorporated with (a) 1.0 mg/L BAP and (b) 2.0 mg/L BAP respectively (Bar = 1cm)

According to Azad *et al*. (2005), the combination of NAA and BAP resulted in the regeneration of maximum number of adventitious shoots from the leaf-derived callus of *Phellodendron amurense*. In this study, the combination of Napthalene acetic acid (NAA) at 0.1-1.0 mg/L and BAP at 0.5-2.0 mg/L was supplemented in the modified MS medium. Globular structures were detected on the midrib of lamina explants cultured on 0.1 mg/L NAA with 0.5, 1.0 or 2.0 mg/L BAP after three months of initiation culture (Figure 4a & 4b). However, there was neither bud nor shoot regeneration after six months of culture. Tidema and Hawker (1982) reported that shoot regeneration also did not occur in various combinations of BAP and NAA from the callus derived from leaf explants of three *Euphorbia* species (*E. peplus, E. lathyris* and *E. tannensis*).

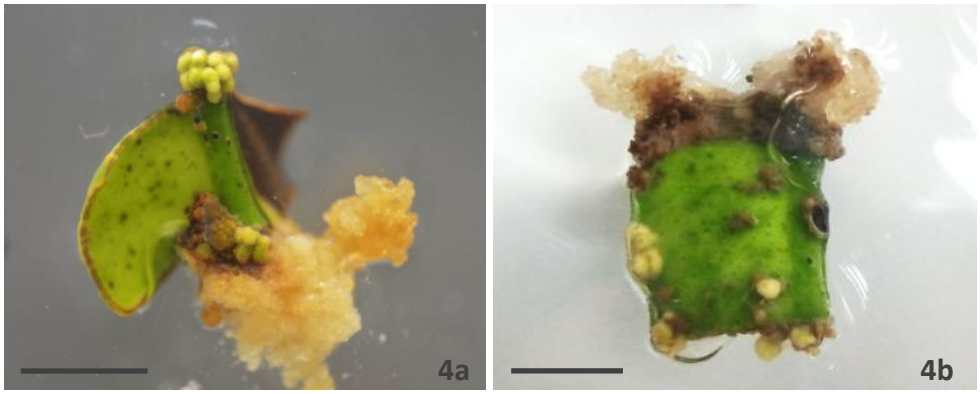

Figure 4 Green globular structures were observed on midrib of lamina explants cultured on modified MS medium supplemented with NAA at 0.1 mg/L in combinations with BAP at (a) 0.5 mg/L and (b) 1.0 mg/L respectively (Bar = 1cm)

Lamina explants cultured on modified MS medium supplemented with Thidiazuron (TDZ) alone in concentration ranging from 0.2-1.0 mg/L developed callus after 10 days of culture regardless of the various concentrations tested (Figure 5a & 5b). However, similar results were obtained as in treatments using BAP and NAA. There was no bud or shoot regeneration directly from the explants cultured on modified MS medium supplemented with TDZ alone.

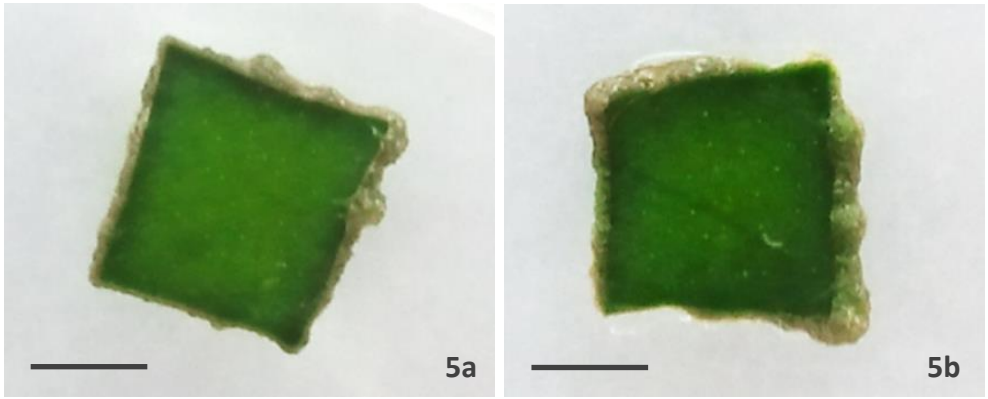

Figure 5 Callus formation was observed on lamina explant cultured on modified MS medium supplemented with TDZ at (a) 0.4 and (b) 0.8 mg/L after 23 days of culture (Bar = 1cm)

V. Indirect Organogenesis

For this study, 2,4-D was applied at 0.2 – 1.0 mg/L for the initial callus induction of lamina explants. The results were satisfactory. It was noted that the callus formation started between day 8 to day 13 of culture at different concentrations of 2,4-D (Table 1). Generally, the calli obtained were white, fluffy and friable in structure with no morphological difference at different concentrations of 2,4-D (Figure 6a & 6b). 2,4-D at 0.4 mg/L produced the highest percentage of explants with callus formation, followed by 2,4-D at 0.2 mg/L. However, statistical analysis showed that there was no significant difference between these two 2,4-D concentrations, i.e. 0.2 mg/L and 0.4 mg/L. Therefore, the optimum concentration of 2,4-D for initial callus induction of indirect organogenesis was 0.2 mg/L.

Table 1 Percentage of explants with callus formation and number of days the callus was first detected on MS medium incorporated with different concentrations of 2,4-D after 64 days of culture

2,4-D concentrations (mg/L)	**0.2**	**0.4**	**0.6**	**0.8**	**1.0**
Callus formation (%)	77.8	**80.6**	72.2	52.8	72.2
Callus first detected (Day)	Day 10	Day 13	Day 10	Day 13	**Day 8**

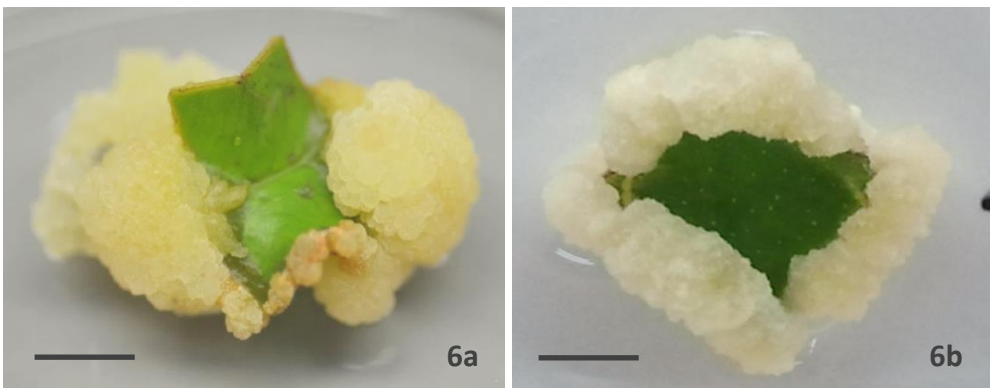

Figure 6 White, fluffy and friable calli formed on MS medium incorporated with 2,4-D at (a) 0.6 and 1.0 mg/L after one month of culture (Bar = 1cm)

Calli obtained were transferred to MS medium added with two types of cytokinins alone, i.e. BAP at 1.0 - 8.0 mg/L and TDZ at 1.0 - 2.0 mg/L. According to Vengadesan *et al*. (2002), adding BAP to the culture medium increased the compactness of callus which is essential for regeneration. However, to date, the addition of BAP or TDZ alone at different concentrations in MS medium failed to regenerate shoots from calli formed.

Besides that, the calli induced were also transferred to MS basal medium supplemented with NAA at 0.2 mg/L and BAP at 5-2.0 mg/L. Although green, globular and compact calli were obtained, no bud or shoot was detected after three months of culture (Figure 7a & 7b). Similar result was also reported by Altaf Hussain *et al*. (2013) where the green callus formed from leaf explant of *Taxus wallichiana* failed to regenerate shoot. Thus, based on the observation, it is concluded that organogenesis was not successful with different combination and concentrations of plant growth regulators studied on Ramin explants.

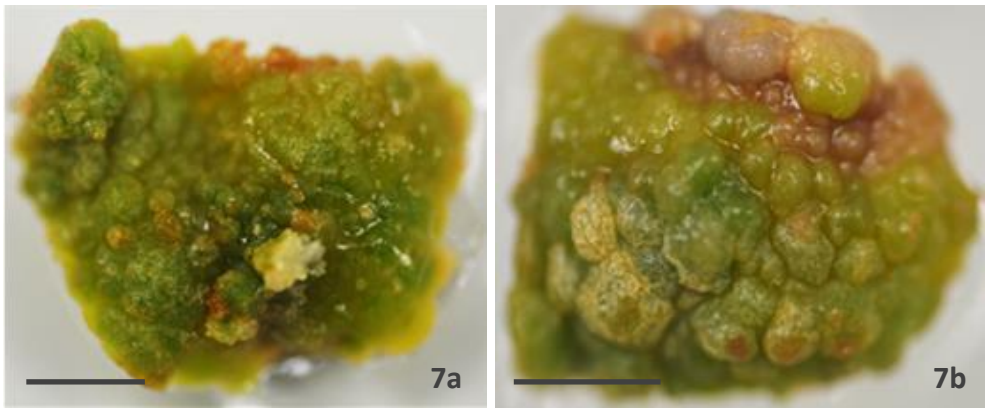

Figure 7 Greenish globular calli cultured on MS medium supplemented with NAA 0.2 mg/L in combination with BAP at (a) 0.5 and (b) 1.0 mg/L respectively (Bar = 1 cm)

VI. Somatic embryogenesis

For plant regeneration by somatic embryogenesis, axenic lamina explants are commonly cultured on basal medium with high concentrations of auxin (George, 1993). In this study, NAA with concentrations ranging from 5 - 30 mg/L were added to both WPM and modified MS media. The effects of NAA at different concentrations on the axenic explants in the two basal media were studied.

Callus formation was observed after seven days of culture. Statistical analysis showed that there was no significant difference for the mean percentage of explants with callus formation on the five different NAA concentrations for both WPM and modified MS basal media (Table 2). Calli developed in WPM medium were yellow, compact and globular regardless of NAA concentrations after three months of culture (Figure 8a – 8d). These calli seemed to have potential in somatic embryogenesis induction. Calli established in WPM medium showed morphological differences compared to those obtained in modified MS medium. This was significant at 20 and 30 mg/L NAA. Calli obtained on modified MS added with NAA at these two concentrations were white and fluffy after three months of culture (Figure 9a, 9b, 10a & 10b). According to Yelnititis & Komar (2010), embryogenic callus was generally friable and nodular, white or yellow in colour on modified MS medium incorporated with 7 mg/L 2,4-D and 1.5 mg/L Biotin. However, they also reported that somatic embryogenesis has yet to be obtained. On the other hand, Das *et al*. (1996) reported somatic embryos formation using WPM medium. Thus, WPM was chosen to be the optimum basal medium for Ramin somatic embryogenesis based on the calli morphology observation.

Table 2 Percentage of explants with callus formation at different concentrations of NAA incorporated in WPM and modified MS respectively after 21 days of culture

NAA concentrations (mg/L)	Basal medium	
	WPM	Modified MS
5	69.4	36.1
10	19.4	50.0
15	41.7	61.1
20	30.6	30.6
30	30.6	33.3
Mean ± SE	38.34 ± 8.53[a]	42.22 ± 5.78[a]

* Value followed by same alphabet is not significantly different using T test at 5% level

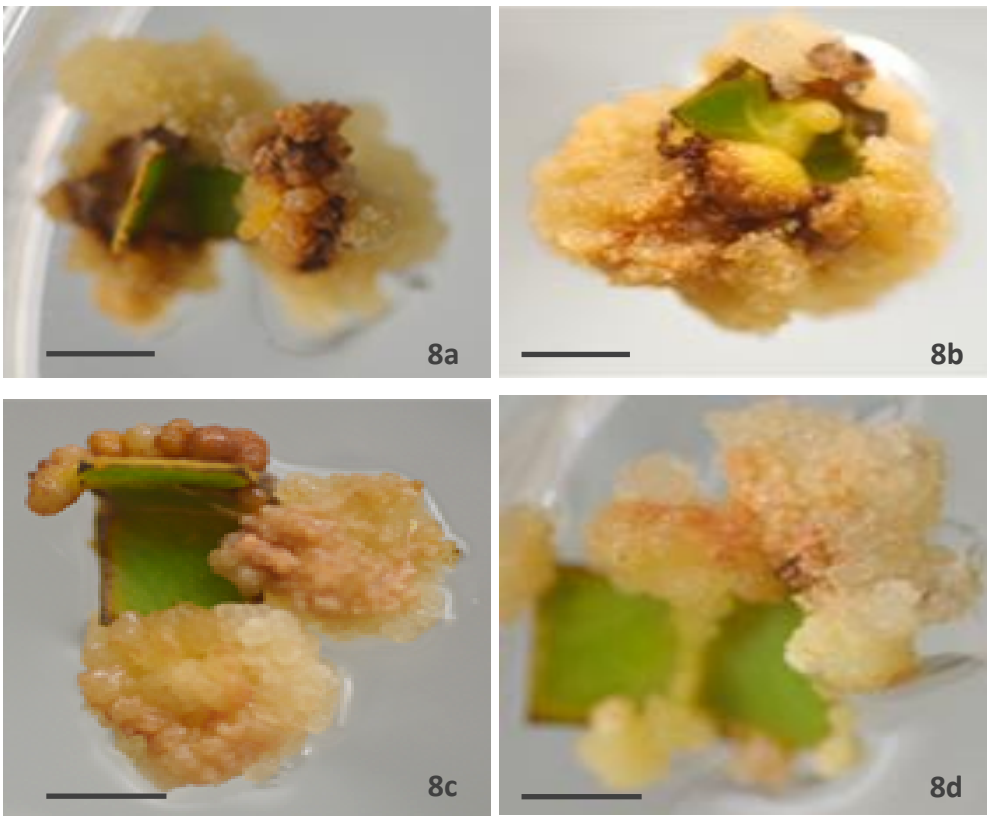

Figure 8 Calli formation on lamina explants after three months of culture on WPM medium supplemented with NAA at (a) 5, (b) 10, (c) 20 and (d) 30 mg/L (Bar = 1 cm)

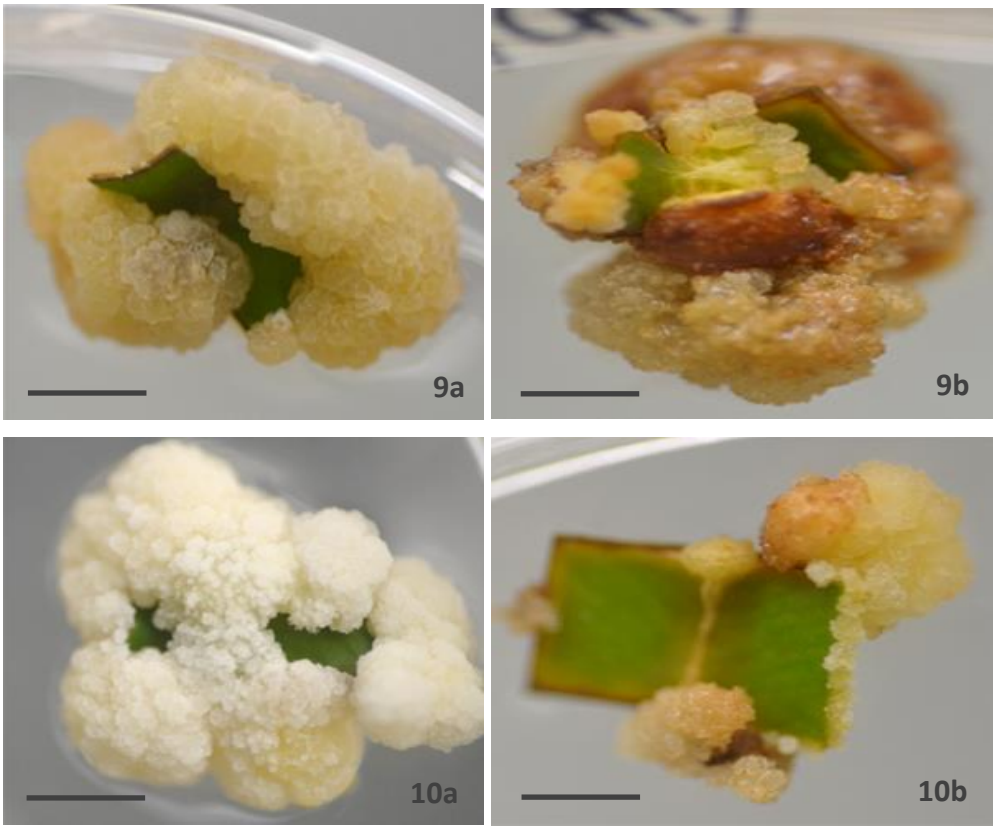

Figure 9 Calli developed on (a) modified MS and (b) WPM incorporated with NAA at 20 mg/L respectively

Figure 10 Calli developed on (a) modified MS and (b) WPM incorporated with NAA at 30 mg/L respectively

The effects of different auxins on somatic embryogenesis were further studied. Axenic lamina explants were cultured on WPM medium incorporated with NAA, 2,4-D and Picloram alone at 5, 10, 15, 20 and 30 mg/L respectively. Picloram induced the earliest callus formation in culture, i.e. day 6, followed by NAA at day 7 and 2,4-D at day 10. Based on the Duncan's Multiple Range Test (DMRT), there was no significant difference on the callus induction on WPM basal medium supplemented with different types of auxins. After 21 days of culture, it was observed that calli induced using different auxins at different concentrations showed no difference morphologically (Figure 11). To date, there was no sign of embryogenic formation.

Table 3 Percentage of explants with callus induced on different auxins at five different concentrations incorporated into WPM medium after 21 days of culture

Concentrations (mg/L)	WPM medium		
	2,4-D	NAA	Picloram
5	41.7	69.4	69.4
10	44.4	19.4	83.3
15	63.9	41.7	47.2
20	83.3	30.6	47.2
30	58.3	30.6	44.4
Mean ± SE	58.32 ± 7.50[a]	38.34 ± 8.53[a]	58.30 ± 7.71[a]

* Value followed by same alphabet is not significantly different using Duncan's new multiple range test at 5% level.

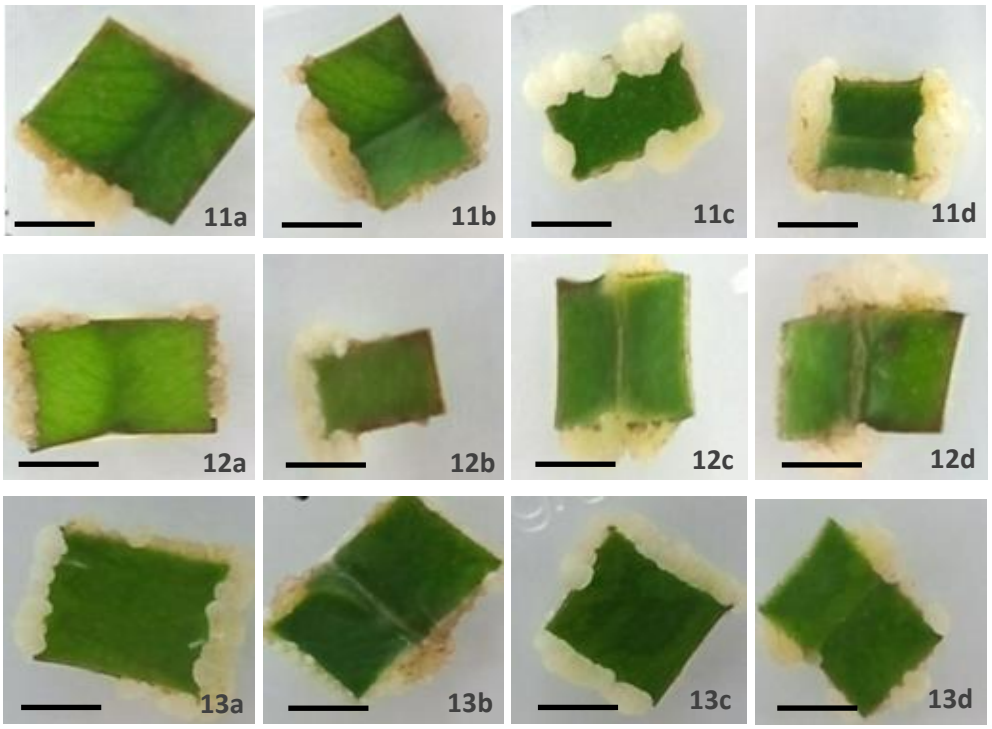

Figure 11 Callus formation on explants cultured on WPM medium supplemented with 2,4-D at 5, 10, 20 after three weeks of culture

Figure 12 Callus formation on explants cultured on WPM medium supplemented with NAA at 5, 10, 20 after three weeks of culture

Figure 13 Callus formation on explants cultured on WPM medium supplemented with Picloram at 5, 10, 20 after three weeks of culture (Bar = 1cm)

The use of NAA for induction of somatic embryogenesis has been reported in *Eucalyptus* species such as *E. citriodora* (Muralidharan & Mascarenhas, 1987; Muralidharan *et al*., 1989), *E. dunnii* (Termignoni *et al*., 1996) and *E. globules* (Pinto *et al*., 2002a). In this study, the effects of NAA at higher concentrations were further studied. NAA at 30, 35, 40, 45 and 50 mg/L was added to the WPM medium. The formation of callus was first observed after 12 days of culture. The highest percentage of explants with callus induced was recorded at 30 mg/L NAA. The calli formed at different NAA concentrations were yellowish-white with compact structures. According to Chen and Chang (2009), embryogenic calli of *Cinnamomum kanehirae* which were induced from young leaves were yellowish-white, compact and granular. Somatic embryogenesis of *Quercus suber* was observed on callus with compact structure (Pinto *et al*., 2002b). Thus, the calli obtained from NAA indicated positive somatic embryogenesis induction.

Table 4 Percentage of explants with callus formation and number of days the callus was first detected on WPM medium incorporated with different concentrations of NAA after 66 days of culture

WPM medium	**NAA concentrations (mg/L)**				
	30	**35**	**40**	**45**	**50**
Callus formation (%)	85.2	76.9	53.7	59.3	58.3
Callus first detected (Day)	Day 12	Day 13	Day 13	Day 13	Day 13

The somatic embryos formation was detected from calli cultured on WPM medium supplemented with 30, 35 and 40 mg/L NAA. Somatic embryos developed on 30 and 35 mg/L NAA were detected after 84 days of culture while embryos cultured on 40 mg/L was observed after six months of culture (Figure 14a, 14b & 14c). Somatic embryos formed were isolated and cultured on fresh medium of the same composition for further proliferation (Figure 15a, 15b & 15c). Unfortunately, after nine months of culture, the

somatic embryos showed no sign of further proliferation and turned brown eventually.

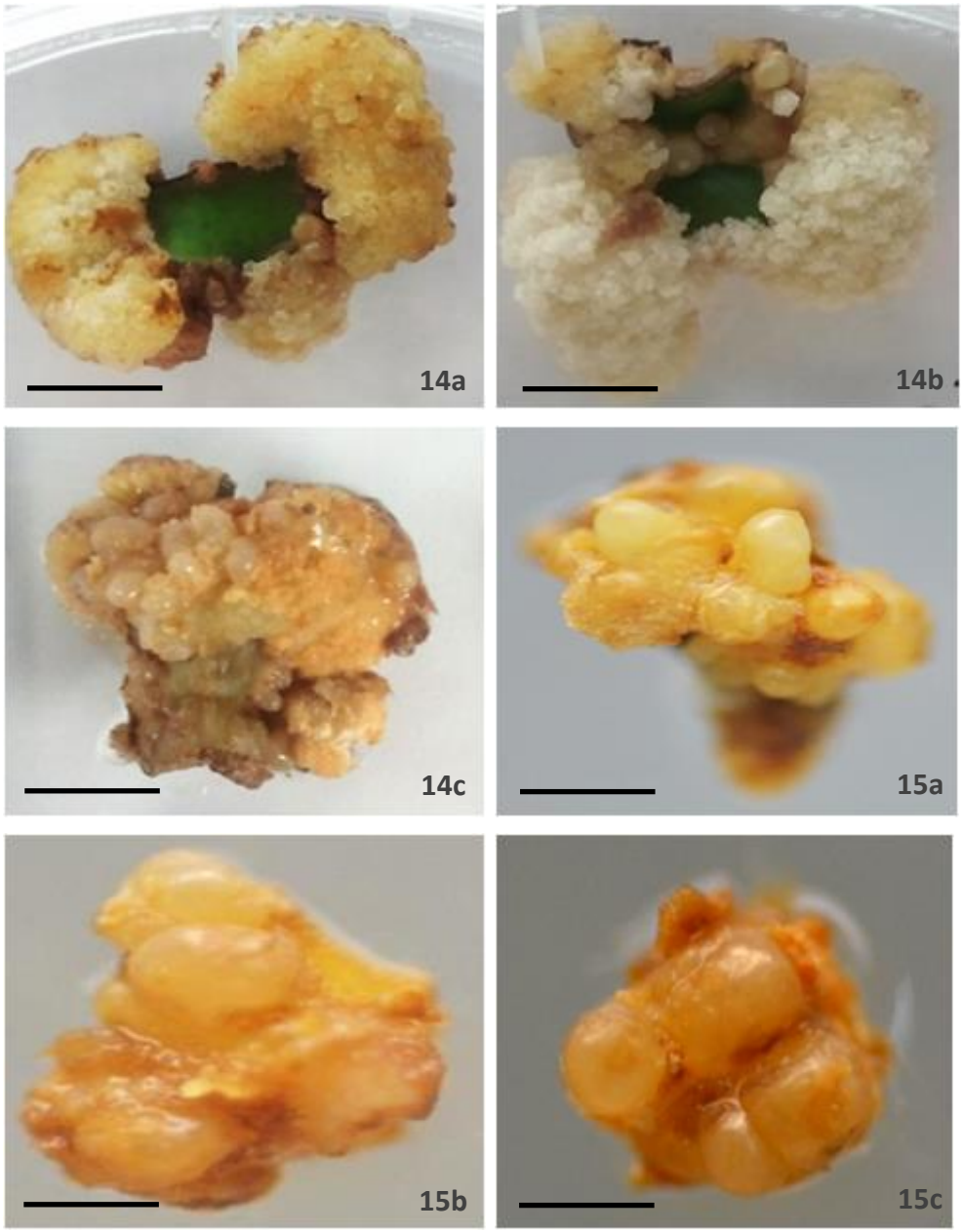

Figure 14 Formation of somatic embryos on WPM medium supplemented with NAA at (a) 30, (b) 35 and (c) 40 mg/L

Figure 15 Somatic embryos isolated from 40 mg/L NAA and further cultured on fresh medium of the same composition

VII. Conclusion

Besides direct shoot induction, two alternative pathways of plant tissue culture, i.e. organogenesis and somatic embryogenesis were applied for the *in vitro* propagation of *G. bancanus* (Ramin) in Sarawak. Generally, based on our observations, different basal medium produced calli of different morphology. The optimum basal medium for direct organogenesis was modified MS, while for somatic embryogenesis, the optimum basal medium was WPM.

For both direct and indirect organogenesis, the use of cytokinins alone or in combination with auxins at different concentrations for shoot induction failed to generate positive results. To date, no sign of bud or shoot growth was detected on the calli obtained.

On the other hand, for somatic embryogenesis, NAA was effective in somatic embryos induction. NAA at high concentrations produced yellowish-white compact calli. Somatic embryos formation was recorded at 30, 35 and 40 mg/L NAA respectively. However, to date, no further proliferation of the somatic embryos was observed. Further studies are therefore required to increase the somatic embryogenesis frequency as well as to induce the globular embryo to further develop and germinate.

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