Axenic Culture Establishment of *Gonystylus bancanus* (Miq.) Kurz (Ramin) In Sarawak

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ITTO-CITES Activity

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

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BAP	6-benzyl amino purine
$HgCL_2$	Mercuric chloride

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Summary

In plant tissue culture, culture contamination is the major problem encountered for the establishment of viable culture using field-grown plant materials. The main objective of this study is to develop effective protocol for the axenic (contamination-free) culture establishment of Ramin in Sarawak. Two different types of sterilants were applied, i.e. Clorox and Mercuric Chloride ($HgCl_2$). Based on the results obtained from this study, the optimum protocols for axenic nodal and shoot-tip explants derived from epicormic shoots are treatments with 0.2% $HgCl_2$ and 0.3% $HgCl_2$ respectively for 5 minutes. For axillary shoots, optimum treatment for axenic shoot-tip explants is 0.2% $HgCl_2$ for 20 minutes. On the other hand, optimum protocol for axenic lamina explants establishment is 0.2% $HgCl_2$ for 10 minutes.

1. Introduction

Gonystylus bancanus (Miq.) Kurz, from the family Thymelaeaceae, is commonly known as ramin. Ramin is a medium-sized to fairly large tree, occasionally attaining 90 cm in diameter at breast height and 45 m height with a clear straight bole of 20 – 30 m (Soerianegara & Lemmens, 1994). This species is native to Indonesia (western and central Kalimantan, south-eastern Sumatra and Bangka), Malaysia (south-western Peninsular Malaysia and Sarawak) and Brunei Darussalam (Hero Dien, 2002). It occurs gregariously in lowland freshwater swamp or peat swamp forests outside the influence of tidal water but often in broad belt along the coast and rarely found above 100 m altitude and is occasionally found in pure stands. It is of high value for light construction and numerous uses where a clear, whitish timber is desired (Hero Dien, 2002). The timber is widely used for decorative cabinet making and furniture (Ng & Shamsudin, 2001).

Ramin is the most important source of *Gonystylus* timbers. It is of good demand, both locally and internationally (Appanah *et al*., 1999), and can be considered as one of the major timbers exported from South-East Asia (Soerianegara & Lemmens, 1994). It is exported to countries such as Japan, China, Taiwan and Hong Kong (Anon, 2000). The Sarawak Forest Department's statistics of ramin exported from the State revealed that the highest volume exported were in the 1960s and 1970s ranging from 602,480 m^3 to 779,651 m^3. However, the production declined to its lowest record in 2000 at 27,042 m^3 (Anon, 2000). In order to fulfill the increasing demand of ramin, Sarawak imported a total of 578,989 m^3 logs and 412,370 m^3 sawn timber from Indonesia, Brunei Darussalam and Sabah from 1970 to 1978. Ramin population reduction is also associated with land development where forested peat lands were cleared and developed for agriculture, aquaculture, industries and residential uses.

Ramin is categorized as vulnerable in the IUCN Red List as its populations and habitats have decreased sharply as a result of over exploitation (Hero Dien, 2002). In an attempt to curb detrimental population loss of this species, it is listed under Appendix II in the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES). Although the Sarawak Government has imposed a ban on ramin logs export which was enforced on 10 May 1980 as a control measure to protect the ramin population in peat swamp forests (Anon, 1980), this ban does not affect the export of ramin timber products. Thus, it is important to improve the productivity of peat swamp forests by planting the degraded areas with ramin in order to sustain their role as an important source of ramin timber. On the other hand, the establishment of ramin plantations in non-peat swamp areas is also crucial to supplement the production from the natural peat swamp forests and as a step towards natural forest conservation. With this, raising sufficient planting materials is an important component for both species recovery and plantation establishment purposes.

Therefore, Ramin propagation has to be done in order to provide sufficient planting materials to be raised for species recovery program or Ramin plantation establishment. Shoot cultures of Ramin by the tissue culture technique using seed has been reported by FRIM (Rosilah *et al*., 2006). The seeds of Ramin are scarce as the flowering is irregular and the fruits are eaten by animals once they dropped on the ground. As seeds are hard to find, it is necessary to use the other parts of the Ramin plant as the source of starting materials for tissue culture i.e. the explant. On the other hand, callus formation of Ramin through tissue culture method was reported by Yelnititis & Komar (2011). Conventional propagation of Ramin by rooting of cuttings is possible but not efficient in producing large number of propagules. Micropropagation is an alternative mode of vegetative propagation which can generate large number of clonal propagules provided the technique has been established.

In plant tissue culture, culture contamination is the major problem encountered for the establishment of viable cultures. Biological contaminants usually refer to the bacteria and fungi found on or within the explants. These contaminants are either due to systemic contaminants or improper use of aseptic laboratory procedures (Cassells, 1991). However, contamination rates vary among tree species and some are tree-specific due to surface peculiarity of the explants (Davis and Keathly 1987). Contaminants elimination is the first stage for *in vitro* propagation using field-grown planting materials. Thus, the main objective of this study is to develop an effective protocol for the axenic culture establishment of Ramin in Sarawak.

2. Plant Materials

2.1 Wildings

Ramin wildings were collected from two different locations in Sarawak, i.e. Lingga, Sri Aman and Loagan Bunut National Park, Miri. The collected wildings were initially kept in the nursery for hardening (Figure 1). A trial planting of ten wildings was initiated in the hedge orchard, however the growth rate was very slow (Figure 2). Bending was carried out on these planted wildings after three months of planting for rejuvenation purpose but they failed to produce epicormic shoots (Figure 3). One of the factors contributing to the failure was the age of the wildings. Wildings collected were young and small in stem size. Thus, the hedge orchard cannot provide adequate explants to be used for this project. On the other hand, the remaining wildings collected were kept in the green house (Figure 4). These wildings were sprayed with 0.2% a.i. Mancozeb once a week. The shoot tips, nodes and leaves of the wildings were pruned to be used as source of explants.

Figure 1 Wildings collected from Lingga, Sri Aman and Loagan Bunut National Park, Miri were potted and kept in the nursery for hardening for two months

Figure 2 Wildings planted in hedge orchard after six months of planting

Figure 3 (a)Bending was carried out on planted wildings in the hedge orchard to induce epicormic shoot formation, (b) but no epicormic shoot sprouted after three months of bending

Figure 4 Wildings kept in the green house to serve as source of explants

The growth of these wildings, both in the hedge orchard and green house, was slow and the number of shoots which could be harvested for use was very limited. Thus it is necessary that a method of bud-forcing be adopted. In this study, two methods of bud forcing were applied, i.e. bending of saplings in field and cuttings.

2.2 Epicormic Shoots through Bending

Bending of saplings was carried out in the field in Lingga, Sri Aman to induce epicormic shoots formation (Figure 5). Epicormic shoots were detected on the stem of bent saplings after three to four months of bending (Figure 6). There were a total of 63 bendings done at different period of time during this project (Table 1). Bending of the saplings was carried out in a staggered manner in order to ensure sufficient supply of planting materials throughout the study period (Table 2). After the collection of samples, the epicormic shoots collected were sprayed with 0.2% a.i. Mancozeb and covered with wet newspapers before being brought back to the laboratory for further experimental work.

Table 1 Number of bendings carried out in Lingga, Sri Aman at different period of time

Batch	Date of Bending	No. of Bending
1	10 October 2012	5
2	20 November 2012	7
3	20 January 2013	15
4	5 February 2013	19
5	26 February 2013	6
6	28 August 2013	9
7	12 September 2013	2
	Total	**63**

Table 2 Number of epicormic shoots available in field and height of epicormic shoots recorded during the assessment period

Date	No. of Epicormic Shoots	Height (cm)
26.02.2013	306	0.10-25.0
12.03.2013	93	0.10-25.0
26.03.2013	163	0.10-34.0
09.04.2013	172	0.10-60.0
23.04.2013	95	0.20-31.0
07.05.2013	124	0.10-40.5
21.05.2013	120	1.00-44.0
25.06.2013	157	0.50-68.2
11.07.2013	210	0.50-58.0
21.07.2013	222	0.70-67.5
15.08.2013	178	0.50-75.0
29.08.2013	256	0.30-43.2
12.09.2013	201	0.50-45.0
25.09.2013	216	0.50-31.3
10.10.2013	217	0.50-45.5
24.10.2013	236	0.50-30.0
07.11.2013	276	0.50-33.0
21.11.2013	240	0.60-39.5
05.12.2013	273	0.50-53.8
20.02.2014	280	0.50-65.0
27.02.2014	296	0.50-55.5
13.03.2014	215	0.50-55.1

Figure 5 Bending of a sapling in Lingga, Sri Aman

Figure 6 Epicormic shoots induced from the bent saplings in the field

2.3 Axillary Shoots through Cuttings

Axillary shoots were induced from cuttings in the green house under mist condition. Branches were collected from the saplings and mature tree of Ramin in Lingga, Sri Aman and Loagan Bunut National Park, Miri. In the field, branches collected were sprayed with 0.2% a.i. Mancozeb and covered with moist newspapers to be brought back to the green house. These branches were then trimmed as cuttings. Cuttings are categorized into two different types, i.e. thin cuttings (diameter < 1 cm) and thick cuttings (diameter > 1 cm). For thin cuttings, some of them were inserted vertically into perlite, the rooting medium (Figure 7a), whereas the remaining thin cuttings together with the thick cuttings were laid horizontally on the perlite (Figure 7b). It took an average of one to two months for the thin cuttings to sprout new axillary buds irrespective to whether they were inserted vertically into or laid horizontally on perlite. For the thick cuttings, emergence of buds was observed after two to three months (Figure 8). After the buds have grown into shoots of about 3 cm long, the shoots were excised from the cuttings and used as source of explants for culture.

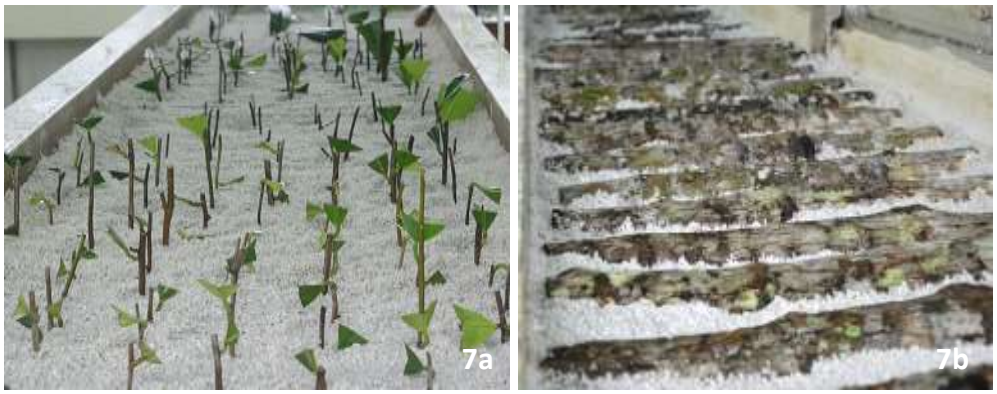

Figure 7 Thin cuttings of Ramin inserted (a) vertically and (b) horizontally onto perlite, the rooting medium

Figure 8 Axillary shoots induced from both the thin and thick cuttings

3. Surface Sterilization: Epicormic Shoots & Axillary Shoots

The epicormic shoots obtained from the field were used as source of shoot-tip and nodal explants for the experiments, while axillary shoots derived from cuttings served as source of shoot-tip explants. These explants were first disinfected using 75% Ethanol with agitation for one minute, followed by Mercuric chloride ($HgCl_2$) at three different concentrations, i.e. 0.2, 0.3 and 0.4% for 5, 10 and 20 minutes respectively. The sterilant was then decanted and the explants were rinsed five times with sterilized-RO water for three minutes respectively. Explant cut ends which were damaged by the sterilant were trimmed off before culturing on RAM medium.

3.1 Epicormic Shoots

After three days of culture, browning was observed at the top part of the shoot-tip explants. Contamination was detected after six days of culture. Based on the result obtained at day 21 of culture, treatments with 0.2% $HgCl_2$ for 10 minutes as well as 0.3% $HgCl_2$ for 5 minutes recorded 30% of axenic shoot-tip explants. For nodal explants, treatment with 0.2% $HgCl_2$ for 5, 10 and 20 minutes obtained the same percentage of axenic explants, i.e. 20% (Table 3).

Therefore, treatment with 0.3% $HgCl_2$ for 5 minutes was chosen as the optimum surface sterilization regime for shoot-tip explants as less explant were damaged by sterilants compared to treatment with 0.2% $HgCl_2$ for 10 minutes. For nodal explants, treatment of 0.2% $HgCl_2$ at 5 minutes exposure time was suggested as the optimum sterilization regime. Statistical analysis indicated that there was no significant difference in the axenic explants obtained for both nodal and shoot-tip explant respectively at different concentrations and exposure time of $HgCl_2$. The axenic explants obtained

were then transferred to media supplemented with 6-benzyl amino purine BAP for induction of shoot proliferation.

Table 3 Percentage of contaminated, axenic and damaged shoot-tip and nodal explants at different concentrations and exposure time of $HgCl_2$ at day 21 of culture

Explants	Time (minutes)		Mercuric Chloride Concentrations (%)		
			0.2	0.3	0.4
Shoot Tips	**5**	**Axenic**	20	30	10
		Contaminated	70	60	40
		Damaged	10	10	50
	10	**Axenic**	30	10	20
		Contaminated	30	60	10
		Damaged	40	30	70
	20	**Axenic**	20	0	0
		Contaminated	10	10	0
		Damaged	70	90	100
Nodes	**5**	**Axenic**	20	10	10
		Contaminated	80	90	60
		Damaged	0	0	30
	10	**Axenic**	20	10	10
		Contaminated	80	70	40
		Damaged	0	20	50
	20	**Axenic**	20	0	0
		Contaminated	50	70	0
		Damaged	30	30	100

3.2 Axillary Shoots

Browning of the shoot-tip explants was observed after 13 days of culture. Treatment with 0.2% $HgCl_2$ for 20 minutes marked the highest percentage of axenic explants obtained after 21 days of culture initiation. For 0.3% $HgCl_2$ at 5 and 10 minutes, both treatments produced 43% axenic explants respectively. The explants treated with 0.3% $HgCl_2$ for 10 minutes recorded higher percentage of damaged explants compared with that treated for 5

minutes. Treatment with $HgCl_2$ at 0.4% was too harsh for the explants as they turned brown and eventually died after the treatments at three different exposure times, i.e. 5, 10 and 20 minutes. Similar results were obtained for treatment with 0.3% $HgCl_2$ for 20 minutes where 100% damaged explants was recorded (Table 4). Therefore, 0.2% $HgCl_2$ with 20 minutes exposure time was recommended for the surface sterilization of explants derived from axillary shoots. Besides achieving the highest number of axenic explants, this treatment produced the least number of damaged explants.

Table 4 Percentage of contaminated, axenic and damaged shoot-tip explants at different concentrations and exposure time of $HgCl_2$ at day 21 of culture

Time (minutes)		**Mercuric Chloride Concentrations (%)**		
		0.2	**0.3**	**0.4**
5	**Axenic**	14	43	0
	Contaminated	29	14	0
	Damaged	57	43	100
10	**Axenic**	29	43	0
	Contaminated	0	0	0
	Damaged	71	57	100
20	**Axenic**	57	0	0
	Contaminated	14	0	0
	Damaged	29	100	100

4. Surface Sterilization: Lamina

Two different types of sterilants were applied for the establishment of axenic lamina explants, i.e. commercial bleach, Clorox and $HgCl_2$ at different concentrations and exposure time.

4.1 Clorox

Lamina explants were surface-sterilized with Clorox at 20, 30 and 40% for 5, 10 and 20 minutes respectively. Treatment with 30% Clorox for 20 minutes exposure time recorded the highest percentage of axenic explants after 21 days of culture, i.e. 76%, whereas, treatment with 20% Clorox for 10 minutes recorded the highest percentage of contaminated explants, i.e. 64% (Table 5). Statistical analysis showed that there was no significant difference between the Clorox concentrations and exposure time at 5% significance level. With this, treatment with Clorox at 20% for 5 minutes was recommended as the optimum sterilization regime among all the Clorox treatments tested.

Table 5 Percentage of contaminated, axenic and damaged lamina explants at day 21 after initiation of culture treated with different Clorox concentrations and exposure times

Time (minutes)		Clorox Concentrations (%)		
		20	30	40
5	**Axenic**	62.0	52.8	69.5
	Contaminated	38.0	47.2	30.5
	Damaged	0	0	0
10	**Axenic**	36.0	46.2	45.2
	Contaminated	64.0	53.8	52.0
	Damaged	0	0	2.8
20	**Axenic**	39.8	76.0	58.5
	Contaminated	60.2	24.0	41.5
	Damaged	0	0	0

* 3 replicates/treatment, 36 explants/replicate

4.2 Mercuric Chloride ($HgCl_2$)

On the other hand, the effects of $HgCl_2$ on the establishment of axenic lamina explants were studied. After 21 days of culture, treatment with 0.2% $HgCl_2$ for 20 minutes recorded the highest percentage of axenic lamina explants, i.e. 58.3%, while explants treated with 0.3% $HgCl_2$ for 5 minutes recorded the lowest percentage of axenic explants, i.e. 22.3% (Table 6). Analysis of variance showed that there was no significant difference in the interaction between $HgCl_2$ factor and exposure time factor for axenic lamina explants at 5% of significance level. $HgCl_2$ at 0.2% was chosen as the optimum concentration for this study. For exposure time, contamination of explants decreased with the increase of exposure time in $HgCl_2$. Treatment at 5 and 20 minutes was different statistically, but there was no significant difference between treatments at 10 and 20 minutes. Therefore, it was concluded that 0.2% $HgCl_2$ and 10 minutes was the optimum surface sterilization regime in this study.

Table 6 Percentage of contaminated, axenic and damaged lamina explants at day 21 after initiation of culture treated with different $HgCl_2$ concentrations and exposure times

Time (minutes)		Mercuric Chloride $HgCl_2$ (%)		
		0.2	**0.3**	**0.4**
5	**Axenic**	24.2	22.3	31.5
	Contaminated	74.9	76.8	68.5
	Damaged	0.9	0.9	0
10	**Axenic**	42.7	40.7	36.1
	Contaminated	55.5	55.6	63.0
	Damaged	1.8	3.7	0.9
20	**Axenic**	58.3	54.6	56.5
	Contaminated	36.1	31.5	36.1
	Damaged	5.6	13.9	7.4

* 3 replicates/treatment, 36 explants/replicate

4.3 Clorox vs Mercuric Chloride ($HgCl_2$)

A study comparing between the two selected treatments of Clorox and $HgCl_2$ was further conducted. Lamina explants were surface-sterilized with 20% Clorox for 5 minutes and 0.2% $HgCl_2$ for 10 minutes respectively. Treatment with 0.2% $HgCl_2$ for 10 minutes recorded higher percentage of axenic lamina explants (67.5%) compared to treatment with 20% Clorox for 5 minutes (51%)(Table 7). Statistical analysis showed that there was no difference between the two treatments. With this, treatment with 0.2% $HgCl_2$ for 10 minutes was the optimum sterilization regime for lamina explants.

Table 7 Percentage of contaminated, axenic and damaged lamina explants at day 21 after initiation of culture treated at 20% Clorox for 5 minutes and 0.2% $HgCl_2$ for 10 minutes

	Treatments	
	20% Clorox 5 minutes	**0.2% $HgCl_2$ 10 minutes**
Axenic	51.0	67.5
Contaminated	48.0	31.5
Damaged	1.00	1.00

* 3 replicates/treatment, 36 explants/replicate

5. Conclusion

Bending of Ramin saplings was found to be an effective method for rejuvenation to produce sufficient juvenile materials for *in vitro* propagation of Ramin throughout this study. An alternative method to produce juvenile materials was from stem cuttings using branches to produce axillary shoots. Both these rejuvenation techniques are non-detrimental to the Ramin mother plants in the field compared to coppicing. Thus, these two techniques are strongly recommended for the mass production of plant materials for Ramin propagation work in the future.

The primary objective of surface sterilization for Ramin is to eliminate completely the contaminants on the explants to obtain axenic explants *in vitro* for further induction of multiple shoots formation. In this study, two different types of sterilants were used, i.e. Clorox and Mercuric Chloride ($HgCl_2$). Based on the results obtained from this study, effective protocols for the establishment of axenic explants have been developed. The optimum protocols for axenic nodal and shoot-tip explants derived from epicormic shoots are treatment with 0.2% $HgCl_2$ and 0.3% $HgCl_2$ respectively for 5 minutes. For axillary shoots, the optimum treatment for axenic shoot-tip explants is 0.2% $HgCl_2$ for 20 minutes. On the other hand, the optimum protocol for axenic lamina explants establishment is 0.2% $HgCl_2$ for 10 minutes. Axenic nodal and shoot-tip explants obtained could be further transferred for direct shoot induction, while, axenic lamina explants could be used for organogenesis and somatic embryogenesis study.

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