

**Shoot Induction 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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List of Abbreviations

BAP	6-benzyl amino purine
GA	Gibberellic acid
HgCL₂	Mercuric chloride
IBA	Indole-3-butyric acid
MS	Murashige and Skoog medium
NAA	Naphtalene acectic acid

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Summary

Micropropagation serves as an alternative technique to the conventional propagation of Ramin. To date, only two reports on the preliminary findings of Ramin shoot culture have been published. However, no conclusive result on Ramin micropropagation was reported. This study aims to develop viable shoot culture followed by multiple shoots induction in order to continue the effort of developing a suitable protocol for micropropagation of Ramin. Field-grown juvenile planting materials were induced through bending of Ramin saplings (epicormic shoots) as well as from stem cuttings (axillary shoots). Planting materials were surface-sterilized with 75% Ethanol for one minute followed by 0.3% Mercuric Chloride (HgCl_2) for 5 minutes. Axenic shoot-tip and nodal explants obtained were transferred to fresh media added with 6-Benzyl amino purine (BAP) alone and in combination with Naphthalene acetic acid (NAA) for shoot induction. BAP alone was found to be effective in inducing shoot formation on both shoot-tip and nodal explants. Generally, only one shoot was induced from each axenic shoot-tip and nodal explants in the culture. One shoot-tip explant sprouted two shoots at 2.5mg/L BAP after 71 days of culture. Shoots obtained were further transferred to Indole-3-butyric acid (IBA) for rooting, but no sign of root growth was observed.

I. Introduction

Gonystylus bancanus (Miq.) Kurz. (Ramin), a slow growing species thrives in lowland freshwater swamp or peat swamp forest which is rarely found above 100 m a.s.l, and can be found in Malaysia, Indonesia (Kalimantan, Sumatera) and Brunei Darussalam (Kartiko, 2002). This species is one of the valuable tropical timbers traded internationally. The great demand for Ramin from both local and international markets (Appanah *et al.*, 1999) has led to the overexploitation of this species. Due to the significant reduction of its populations, it has been classified as vulnerable in the IUCN Red List of Threatened Species. In addition, the Convention on International Trade of Endangered Species of Wild Flora and Fauna (CITES) has listed Ramin in Appendix II in 2005 (Chen, 2007).

Under natural conditions, new stands of Ramin trees are derived from germinated seeds that fall on the ground, referred to as wildings. As the flowering and fruiting of Ramin is irregular and unpredictable, it is not feasible to rely solely on seeds for raising large quantity of Ramin seedlings. Thus, the possibility of vegetative propagation of Ramin through rooting of cuttings was explored. According to Ismail & Shamsudin (2003) and Mohamad Lokmal *et al.* (1992), Ramin could be propagated vegetatively by cuttings with a rooting ability of more than 50%. Under a mist propagation system, Guanih (2005) obtained 65 to 80% rooting for this species. Moreover, Sumbayak and Komar (2008) showed that more than 90% rooting could be achieved under glass house condition with the humidity maintained at 95% and the temperature kept below 30°C for young stem cuttings taken from the Ramin wildings. Vegetative propagation through rooting of cuttings although successful, may not be an efficient method to supply large number of planting materials required for the rehabilitation of degraded peat swamp forests, unless a hedge orchard (stock plant nursery

or source bush) is established to supply the required amount of young stem cuttings for rooting.

Another form of vegetative propagation using plant tissue culture technique, which is also known as Micropropagation, has been considered and attempted. Shamsudin & Aziah (1992) cited in Khali Aziz Hamzah *et al.* (2010) were of the opinion that Ramin has the potential to produce plantlets through tissue culture. Among the various plant tissue culture techniques for micropropagation, shoot culture is the most direct pathway of plant regeneration and usually the first to be attempted. To date, only two reports on the preliminary findings of Ramin shoot culture have been published. In Indonesia, Yelnititis & Komar (2011) carried out *in vitro* culture of single-node stem explant obtained from seedlings. They found that the emergence of shoots was faster in explants cultured in a medium incorporated with 2.0 mg/L Thidiazuron and the addition of 6-Benzylamino purine (BAP) at 0.5 mg/L was the best for shoot growth. This work is still on-going. On the other hand, in Malaysia, Rosilah *et al.* (2006) cultured emerging shoots from *in vitro* germinated-seedlings in MS medium incorporated with three levels of BAP. They reported that two shoots were observed in the culture of a single shoot explant in MS medium supplemented with 0.5 mg/L BAP.

To continue the effort of developing a suitable protocol for the micropropagation of Ramin, this study aims to establish viable shoot culture followed by induction of multiple shoots formation in the axenic shoot obtained.

II. Plant Materials

Juvenile materials are generally regarded as more amenable for plant regeneration than mature materials. Two types of juvenile materials were used in this study, namely epicormic shoots and axillary shoots. Epicormic shoots were obtained through the bending of Ramin wildings in the field (Figure 1a & 1b), while axillary shoots were established through cuttings in the green house (Figure 2).



Figure 1 (a) Bent Ramin wildings in the field sprouted (b) juvenile epicormic shoots after 16 weeks of bending

Figure 2 Axillary shoots induced from cuttings laid horizontally on perlite after eight weeks in the green house

III. Surface sterilization

The shoots collected from the field and green house were first trimmed to 5 cm-sections. The explants were first disinfected using 75% Ethanol by agitating them for one minute. Subsequently, the explants were sterilized with 0.3% Mercuric chloride (HgCl_2) for five minutes. The sterilant was then decanted and the explants were rinsed five times with sterilized-RO water for three minutes. The cut ends of the explants damaged by the sterilants were trimmed off before culturing on the culture medium.

IV. Shoot culture

Axenic nodal and shoot-tip explants obtained were initially cultured on two types of shoot proliferation basal media, i.e. MS (Murashige and Skoog, 1962) and modified MS medium. Based on our observation, explants cultured on both basal media showed axillary bud emergence after one to two weeks of culture initiation (Figure 3). Only one axillary shoot sprouted from each explant. The axillary shoots exhibited slow growth. However, after 18 weeks of culture, there was no further growth or development on the axillary shoots obtained. This was probably due to the natural slow growth of Ramin as reported by Sukardi & Sutiyono (1994) and the depletion of nutrient reserve in the explant as the new bud or shoot was not in direct contact with the culture medium.

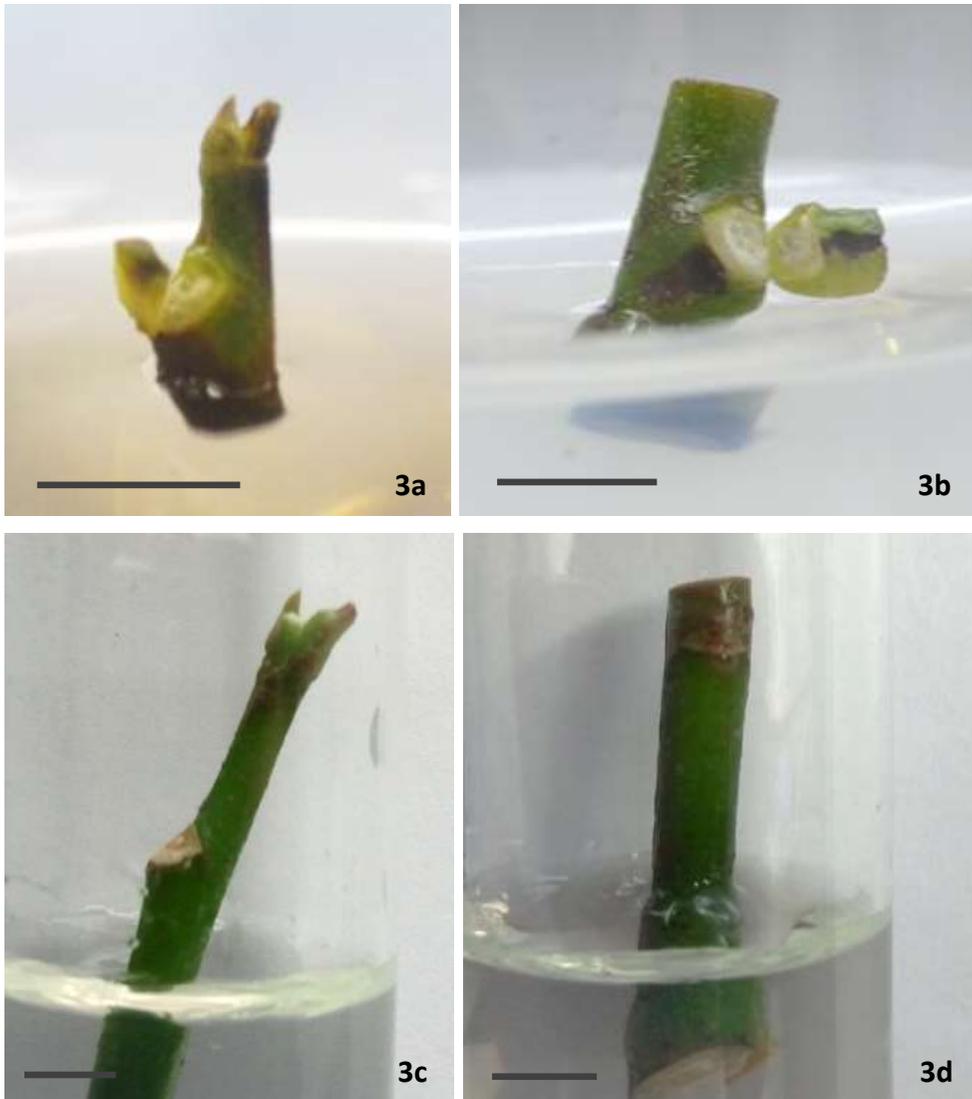


Figure 3 Shoot-tip and nodal explants cultured on (a & b) MS and (c & d) modified MS media respectively after one week of culture initiation (Bar = 5 mm)

Plant growth regulators are known to produce positive effects in plant growth and development. In plant tissue culture study, cytokinin is usually incorporated into the culture medium for multiple shoots induction. Multiple shoots formation was obtained from shoot explants of *Aquilaria agallocha* (He *et al.*, 2005) and nodal explants of *Balanites aegyptiaca* (Rathore *et al.*, 2004) cultured on medium incorporated with BAP. For this study, BAP at 0.5,

1.0 and 2.5 mg/L was supplemented respectively into the two basal media, i.e. MS and modified MS media. The effect of different BAP concentrations on shoot proliferation was investigated. It was noted that shoot-tip explants sprouted axillary buds after one week of culture, while nodal explants sprouted axillary buds after two weeks of culture in the media regardless of the BAP concentrations in the media (Figure 4 & 5). The results showed that there was no difference on the time of axillary bud emergence for explants cultured on the basal media alone or supplemented with BAP.

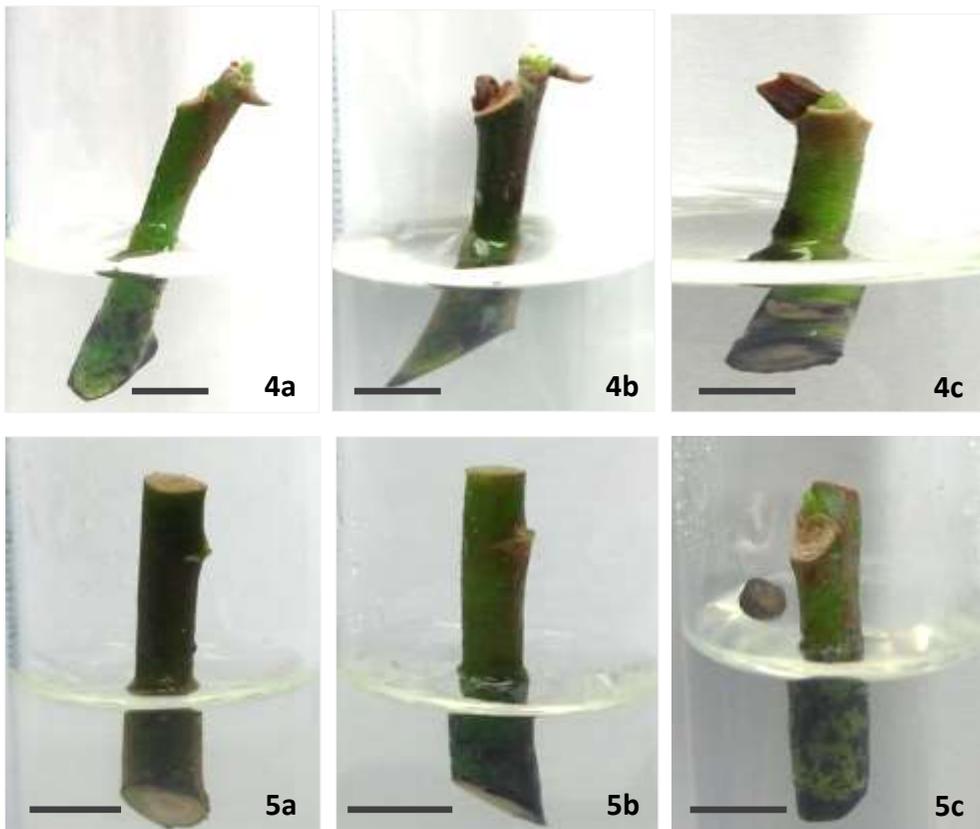


Figure 4 Axenic shoot-tip cultured on basal medium supplemented with BAP at (a) 0.5, (b) 1.0 and (c) 2.5 mg/L respectively after two weeks of culture
Figure 5 Axenic nodal cultured on basal medium supplemented with BAP at (a) 0.5, (b) 1.0 and (c) 2.5 mg/L respectively after two weeks of culture (Bar = 5mm)

Generally, only one single new shoot developed on each of the shoot-tip and nodal explants after the sprouting of axillary bud irrespective of the different BAP concentrations incorporated in the media (Figure 6). These findings are consistent with the research results reported by Yelnititis and Komar (2011) where only one shoot regenerated from each of the single-node explants in spite of the different types and concentrations of cytokinins tested. However, in our study, it was observed that one of the shoot-tip explants successfully sprouted two shoots on the modified MS medium supplemented with 2.5 mg/L BAP after 71 days of culture (Figure 7a & 7b). No multiple shoot formation was detected on shoot-tip explants cultured on media containing 0.5 and 1.0 mg/L BAP after three months of culture.



Figure 6 (a, b & c) Shoot-tip and (d, e & f) nodal explants developed a single new shoot after two to three months of culture on the shoot proliferation media (Bar = 5mm)



Figure 7 (a) Two shoots were induced from one of the shoot-tip explants cultured on modified MS added with 2.5 mg/L BAP at day 71 of culture. (b) Enlargement of figure (a) (Bar = 5mm)

According to Wachira (1997), interaction of BAP and Naphthelene acetic acid (NAA) on shoot regeneration of *Eucalyptus grandis* was statistically significant where shoot regeneration increased with the increase of BAP concentration. The combination of BAP and NAA was found to be the best for both the formation and multiplication of shoots in one of the medicinal plant and Red Sandalwood as reported by Aamir Ali *et al.* (2012) and Warakagoda & Subasinghe (2013) respectively. Thus, in this study, BAP ranging from 0.5 to 2.0 mg/L was combined with NAA at 0.05 to 0.2 mg/L in the modified MS basal medium to induce shoot proliferation. From our observation, there was no marked difference in terms of the number of shoots induced with different combinations of BAP and NAA. In addition, no multiple shoot was induced in all the treatments.

The growth of Ramin shoots induced was slow. Thus, Gibberellic Acid (GA) was added in order to stimulate growth of the explants through its effect on cell division and elongation. Shoot elongation was achieved when explants cultured on medium supplemented with GA in Northern Red Oak

(Vengadesan & Pijut, 2009). In this study, GA, ranging from 0.05 to 0.2 mg/L, was incorporated into the culture media. The sprouted axenic shoot elongated after the transfer to fresh media added with 0.2 mg/L GA, but the explants turned brown and died eventually after 16 weeks of culture. It is believed that the growth of sprouted shoot relied on the food reserve of the explants itself instead of the plant growth regulator added. According to Pierik (1997), the addition of nutrients and hormones has less effect on larger explant as bud or shoots induced will rely on its internal food supply and hormone.

V. Root induction

The shoots induced were further transferred to the root induction media. Indole-3-butyric-acid (IBA) was incorporated in the media with concentrations ranging from 0.5 to 2.0 mg/L. According to Warakagoda and Subasinghe (2013), rooting of explants was successfully induced using IBA in the medium for Red Sandalwood. In this study, callus formation was found at the cut end of both the shoot-tip and nodal explants cultured on 2.0 mg/L IBA (Figure 8a & 8b), while explants cultured on 0.5 mg/L IBA swelled without callus being detected (Figure 9) after two months of culture. Vengadesan & Pijut (2009) also observed more callus formation at the base of shoot cultured on higher concentration of IBA.

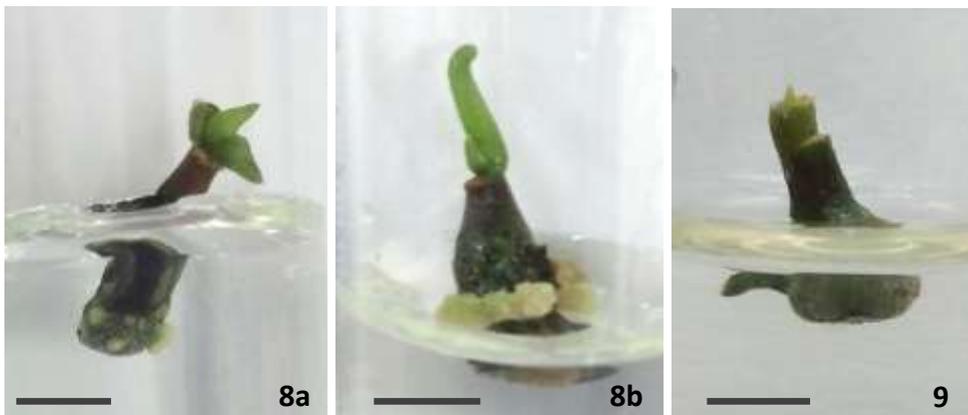


Figure 8 (a & b) Callus detected at the base of shoot-tip explants cultured on modified MS medium supplemented with 2.0 mg/L IBA

Figure 9 Swelling of shoot-tip explant at the cut end, cultured on modified MS medium incorporated with 0.5 mg/L IBA (Bar = 5 mm)

VI. Conclusion

Plant growth regulators, i.e. BAP, NAA and GA were used to stimulate shoots formation and growth from the explants. The results of the present study showed that shoot-tip and nodal explants, cultured on both MS and modified MS basal media added with BAP alone or in combinations with NAA at different concentrations, generally developed only one single new shoot after one to two weeks of culture. Similar observations were obtained for explants cultured on both MS and modified MS basal media without the addition of any plant growth regulator. Only one shoot-tip explants cultured on modified MS medium supplemented with 2.5 mg/L BAP sprouted two shoots after 71 days of culture. Shoots obtained were further transferred to fresh media added with IBA for rooting, but no sign of root growth was observed. Calli developed at the cut ends of the explants at high concentrations of IBA.

Contamination was the major problem associated with the explants derived from field-grown materials. Due to the limited number of axenic explants available, the results obtained were inconclusive. Further work needs to be done to obtain a satisfactory conclusion for this study, especially on the establishment of high quantity of axenic explants as well as shoot induction treatments using different types of cytokinins and auxins.

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