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Full Length Research Paper

Effect of 6-benzylaminopurine (BAP) in different basal media on shoot multiplication of *Aquilaria hirta* and detection of essential oils in the *in vitro* shoots

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***Aquilaria hirta*, locally known as 'chandan' or 'chandan bulu', is used interchangeably with *Aquilaria malaccensis* (karas) to produce gaharu. With the decline in the *A. malaccensis* populations, harvesting of this precious wood has begun to extend to *A. hirta*. There is also an increasing interest to plant karas trees and related species. Therefore, a rapid propagation method is necessary to meet the increasing demand for planting materials as well as for conservation purposes. In this paper, we reported on the effect of 6-benzylaminopurine (BAP) in different basal media, Murashige and Skoog (MS) (1962) and woody plant medium (WPM) on shoot multiplications of *A. hirta* using nodal segments from young seedlings of *A. hirta* as explants and the chemical analyses of essential oils present in the *in vitro* shoots. The MS medium supplemented with 0.1 mg/l BAP was the most effective for multiple shoot formation in *A. hirta*, producing an average of 6.1 shoots per culture, which is significantly higher than other treatments. New shoots produced attained a length of 0.4 to 1.8 cm within 25 days in both MS and WPM basal media. The highest length of new shoots was obtained in WPM containing 0.1 mg/l BAP. Analysis of the oils by gas chromatography (GC) and gas chromatography-mass spectrometry (GCMS) revealed the presence of β-caryophyllene in trace amounts. Other compounds detected were tetradecanal, hexadecanoic acid, methyl linoleate, linoleic acid, isophytol and phytol acetate.**

Key words: *Aquilaria hirta*, Chandan, shoot multiplication.

INTRODUCTION

Gaharu, also known as agarwood or eaglewood, is a highly valuable fragrant wood used for incense, perfume and traditional medicines (Soehartono and Newton, 2000). Gaharu is derived principally from tropical trees of the genus *Aquilaria* Lam. (Thymelaeaceae). The genus *Aquilaria* is distributed in southern Asia from India to China and throughout most of Southeast Asia (Whitmore, 1972). It is derived from the resinous substance produced by the tree in response to attack by pathogenic fungi (Ng et al., 1997), although the precise nature of this response is poorly understood (Soehartono and Newton, 2000). The strong heavy scent of gaharu is unique and complex.From the wood, the scent is only slight at room temperature but becomes stronger when the wood is burnt. The Arabs and Japanese enjoy the strong fragrance and incorporate the essence in incense products. Gaharu is also sought after for its medicinal properties (Okugawa et al., 1993). Tissue culture techniques as a means for conserving and multiplying medicinal plants have been reported by Joshi and Dhar (2003), Fracaro and Echeverrigaray (2001) and Borthakur et al. (2000) with the aim for large scale production of plant material.

Phytochemical studies of gaharu from *Aquilaria malaccensis* and other species have shown the presence of sesquiterpenes, sesquiterpene alcohols, oxygenated compounds, acids and several chromone derivatives (Ishihara et al., 1991; Ishihara et al., 1993; Ng et al., 1997; Nor Azah et al., 2006, 2008; Tamuli et al., 2005). Gaharu is traded worldwide in significant volumes. As a

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Abbreviations: MS, Murashige and Skoog; **WPM,** woody plant medium; **BAP,** 6-benzyladeninepurine; **GC,** gas chromatography; **GCMS,** gas chromatography-mass spectrometry.

Table 1. Shoot multiplication and shoot length in MS and WPM basal media derived from young seedlings of *A. hirta* after five weeks of culture.

BAP (mg/l)	MS basal		WPM basal	
	Mean shoot number per explants	Mean shoot length (cm)	Mean shoot number per explants	Mean shoot length (cm)
0.1	6.1[a]	0.5[ab]	1.0[a]	1.8[a]
0.5	4.9[ab]	0.4[b]	3.0[a]	1.7[a]
1.0	4.1[bc]	0.5[ab]	2.8[a]	1.2[ab]
2.5	2.9[cd]	0.7[ab]	3.2[a]	0.8[b]
5.0	2.6[d]	1.1[a]	1.2[a]	0.9[b]

[a] Mean values followed by the same letters in a given column are not significantly different (P = 0.05) according to Duncan's test. WPM, woody plant medium; BAP, 6-benzyladeninepurine; MS, Murashige and Skoog.

direct result of this commercial activity, *Aquilaria* spp. has become the focus of increasing conservation concern (Ng et al., 1997; Soehartono and Newton, 2000). Most, if not all, gaharu being traded are collected from the natural forest. Because of this commercial trade, *Aquilaria* spp. in gaharu-producing countries such as Indonesia, India and Malaysia are reported as being in decline (Soehartono and Newton, 2000).

There are a total of 19 spp. of agarwood native to Malaysia (Peninsula: 13 spp., Sabah: 11 spp., Sarawak: 15 spp.). They have been listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora in effect from January 2005. This means that not only the trade of all species of *Aquilaria* and *Gyrinops* are protected but also any products from these species. The respective agencies are currently monitoring the export of the wood, especially the rampant illegal harvesting of gaharu noted recently in Peninsular Malaysia. Due to the increasing interest in gaharu and the over exploitation of the forests producing it, urgent measures are required to conserve and replenish the plants growing in the wild.

MATERIALS AND METHODS

Shoot multiplication

Shoot tips and nodal segments (1.5 cm) from young seedlings of *Aquilaria hirta* were used as explants. The explants were first washed with tap water and surface sterilized using 50% Chlorox® with 1 drop of Tween 20 for 20 min followed by five rinses in sterile distilled water. The explants were inoculated on MS basal medium containing 0.1 mg/l 6-benzylaminopurine (BAP) for shoot induction. After eight weeks, multiple shoots from culture initiation were removed and individual shoots were excised aseptically. These shoots were subcultured on fresh woody plant medium (WPM) and Murashige and Skoog (MS) basal media supplemented with BAP at concentrations 0.1 to 5.0 mg/l for shoots multiplication. The pH of the medium was adjusted to 5.8 with 1 N NaOH or HCl before autoclaving. All cultures were placed in a culture room at 25°C and 16 h photoperiod under cool white fluorescent light. Each treatment was replicated three times and each replicate consisted of nine explants. Data for shoot multiplication and shoot height were collected after five weeks in culture. The mean number of shoots per explant and mean shoot length were calculated and all data were statistically analysed by Duncan's multiple range tests.

Analysis of essential oil components in *in vitro* shoots

In vitro shoots of *A. hirta* (300 g) were cut into small pieces and subjected to hydrodistillation method in a Clevenger-type apparatus for 6 h. The waxy oily layer obtained was separated and dissolved in n-hexane. The oil yields were calculated based on dry weight of the plant material. Chemical analyses were carried out using gas chromatography (GC) and gas chromatography-mass spectrometry (GCMS). The GC analyses were carried out on a Shimadzu GC-2010 gas chromatograph equipped with a flame ionization detector (FID) using fused silica capillary column CBP-5 (25 m x 0.25 mm; 0.25 µm film thickness); oven temperature programme (60 to 230°C at 3°C/min); Helium as carrier gas and the injector and detector temperature were set up at 220 and 280°C, respectively.

The GCMS analyses were performed on Agilent GCMS 7890A/5975C Series MSD (70eV direct inlet) equipped with HP-5MS fused silica capillary column (30 m x 0.25 mm; 0.25 µm film thickness). The column and injector temperature were the same as those for GC. The mass range was 50 to 550 in the full scan mode with a rate of 2.91 scans/s. The total scan time is 67.7 min. The compounds were identified by matching their mass spectral data with those from the Wiley, HPCH 2205.L and NIST05a.L mass spectral database.

RESULTS AND DISCUSSION

Shoot multiplication

New shoots appeared at the axillary meristem regions of the nodes after one week. In MS basal medium, the highest mean number of new shoots per explant was recorded in 0.1 mg/l BAP (6.1 shoots per culture) and significantly compared with other concentrations ($p < 0.05$). The lowest mean number of shoots was obtained in 5.0 mg/l BAP (2.6 shoots per culture). It was observed that the number of shoots produced decreased as the BAP concentration in the medium increased (Table 1). A reduction in shoot number with increasing cytokinin concentration has also been reported by Joshi and Dhar

Figure 1. Proliferated shoots of *A. hirta* in MS supplemented with 0.1 mg/l BAP.

(2003). BAP alone favours shoot multiplication, as it is considered to be one of the most useful cytokinins for achieving the multiplication and micropropagation of plants (Joshi and Dhar, 2003).

As for shoot elongation, MS containing 5.0 mg/l BAP gave the highest shoot length when compared with other concentrations. Meanwhile in WPM basal medium, the highest frequency of shoot production was observed in 2.5 mg/l BAP (3.2 shoots per culture) but not significantly compared with other concentrations ($p > 0.05$). The lowest frequency of shoot production was observed in 0.1 mg/l BAP (1.0 shoot per culture) but showed the highest shoot length when compared with other concentrations. According to Joshi and Dhar (2003), shoot elongation is retarded in cultures with increased number of shoots.

In this study, it was observed that MS medium favoured the shoots of *A. hirta* than WPM medium for shoot multiplication. Generally, the composition and strength of the basal medium play an important role in shoot multiplication and elongation as well as root induction (Borthakur et al., 2000). This is also applied to *A. hirta* cultures. The difference observed between MS and WPM medium could be attributed to the high content of nitrogen in the MS medium (Fracaro and Echeverrigaray, 2001). The need of MS salts for shoot sprouting and multiplication shows the high salt requirement for the growth of *A. hirta* shoots. Proliferated shoots of *A. hirta* in MS supplemented with 0.1 mg/l BAP is shown in Figure 1.

Analysis of essential oil components in *in vitro* shoots

Essential oils were found to be present in trace amounts (0.01% w/w) from the *in vitro* shoots. The chemical profiles of *A. hirta* in the present study showed marked differences from the chemical components detected from the calli of *Aquilaria crassna* and *Aquilaria sinensis* (Okudera and Ito, 2009). The latter calli were found to contain sesquiterpene hydrocarbons namely α-guaiene, α-humulene and δ-guaiene. However, in this study, it was observed that the *in vitro* shoots of *A. hirta* yielded minor traces of β-caryophyllene, a sesquiterpene hydrocarbon which was not reported in the other species. Other compounds which were present in significant amounts are tetradecanal, hexadecanoic acid, methyl linoleate, linoleic acid, isophytol and phytol acetate ($p < 0.05$). Some of these compounds have been reported in the essential oils of agarwood trees (Tamuli et al., 2005).

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