Establishment of *Aquilaria malaccensis* Callus, Cell Suspension and Adventitious Root Systems

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Abstract

Aquilaria malaccensis is a tropical forest tree from the family *Thymelaeaceae*, an endangered forest species and was listed in CITES since 1995. Locally known as *Pokok Karas*, this tree produces agarwood or *gaharu*, a highly valuable, resinous and fragrant forest product. *Karas* has been highly recognized for its vast medicinal values and *gaharu* has been widely use for perfumery, incense and religious purposes. The phytochemical studies of agarwood showed that Sesquiterpenoid and Phenylethy chromone derivatives are the principal compounds that have anti allergic and anti microbe activities. Cell and organ culture systems provide large scale production of biomass and offers feasibilities for the production of secondary metabolites. This paper describes the work done for establishing reproducible systems for callus initiation and production of cell suspension cultures as well as production of adventitious roots that will later be amenable for the production of secondary metabolites of *A. malaccensis*. Hence, further manipulation with Methyl Jasmonate, a chemical elicitor could be done to induce secondary metabolites using callus, cell suspension and adventitious roots systems.

INTRODUCTION

Aquilaria tree is a tropical forest tree that produces agarwood or *gaharu*, a highly valuable, resinous and fragrant forest product. Agarwood has been highly recognized for its vast medicinal values which have been recorded in the *Sahih Muslim* during the eighth century and also in the *Ayurvedic* medicine. It is also widely use for perfumery, decoration and for religious purposes. It is believed that microbial infection and injury are necessary for the production of the oleoresin compounds (Shimida *et al.*, 1982). The high value of agarwood products also stimulates illegal harvest and trade in various countries. Population of most *Aquilaria* species have declined and are considered threatened due to the overexploitation of agarwood (A. Barden *et al.*, 2002). Due to this unsustainable harvest and trade, one of the species, *A. malaccensis* was listed in Appendix II of the Convention on International Trade in Endangered Species of Wild Fauna and Flora (CITES) with effect from February 1995. Agar wood has complete antibiotic function toward tubercle bacillus, typhoid bacillus and diarrhea bacillus (Wang, 2007). Phytochemical studies of agarwood showed that Sesquiterpenoid (Jantan, 1990) and 2-(2-Phenylethyl) chromones are major constituents and believed to be relevant to the quality of agar wood (Shimida *et al.*, 1986) and have anti allergic activity (Yang, 1998).

Recently, the production of secondary metabolites using plant cells has been the subject of extended research. It was expected that the biosynthetic capacity of plants can be exploited *in vitro* using plant cells and organ systems. The advantage of using plant cell culture as a model system for the study of biosynthetic pathways is because secondary metabolite formation can take place within a short cultivation time (Heike *et al.*, 1995). In many cases, the production of secondary metabolites can be manipulated and enhanced by the treatment of the undifferentiated cells with elicitors such as methyl jasmonate, salicylic acid, chitosan and heavy metals (J. Ebel *et al.*, 1994). With the vast advantages of plant cell cultures and elicitation in producing useful secondary metabolites, we currently conducted a project to look into the identification and production of secondary metabolites namely Sesquiterpens and 2-(2-phenylethyl) chromones compounds in cell suspension cultures of *Aquilaria malaccensis*. In this paper, we reported the studies carried out on the establishment of callus cultures and the establishment of suspension cell cultures of *Aquilaria malaccensis*. Establishment of adventitious roots is also discussed.

1.0 Induction and Multiplication of Callus

Materials and Methods

Plant material, culture media and culture condition

In vitro leaves (Fig. 1) and *in vitro* stem segment of *Aquilaria malaccensis in vitro* plantlets (Fig. 2) were cut into 1x1 cm pieces and cultured onto modified semi-solid Murashige and Skoog Medium (MS medium, 1962) supplemented with various concentration of Dichlorophenoxyacetic acid (2,4-D) ranging from 1.0 mg/l to 10 mg/l and 6-Benzylaminopurine (BAP) at 0.5 mg/l. 30 g/l sucrose and 2.0 g/l gelrite were also added into the media composition. The pH of the media was adjusted to 5.8 before autoclaving for 15 minutes at 121 °C. These cultures were incubated at 24 °C in total darkness and observations on callus induction and proliferation were done every fortnightly.

Results and Discussion

Callus Induction

Callus initiation started after 2 weeks of culture (Fig. 3) and callus proliferation was observed 4 weeks later. Callus formation was observed in all 2,4-D concentrations. Highest percentage of callus formation and proliferation was observed in the lower concentrations of 2,4-D. It was found that MS medium supplemented with 4 mg/l 2,4-D + 0.5 mg/l BAP showed the maximum formation of callus. Similarly, callus and cell proliferation of *A. sinensis* was established with 2,4-D and BAP hormonal combination (Ito *et al.*, 2005). The morphology of the callus formed was whitish/pale yellowish and friable-like structures (Fig. 4). Callus was sub-cultured every fortnightly to avoid browning and for further multiplication by transferring onto fresh media with the same treatment.

Fig.1: *In vitro* plantlets

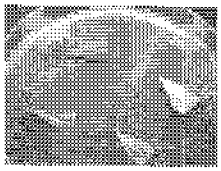

Fig. 2: *In vitro* plantlets

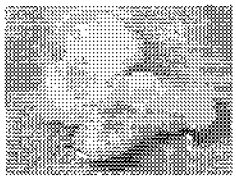

Fig. 4: whitish, friable-like callus

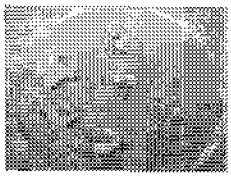

Fig. 3: callus initiation after 2 weeks

Callus multiplication

Callus derived from leaves and stem segments were cultured onto modified MS medium supplemented with low concentration of BAP (1 mg/l) and TDZ (0.5 mg/l). Callus masses maintained in dark condition were routinely sub-cultured every fortnightly to avoid browning and to enhance multiplication. This multiplication process is prerequisite for bulking up of callus, prior for the establishment of cell suspensions and further elicitation treatment. An increment of 5 folds of fresh weight was observed after 6 weeks of culture. Callus clumps was observed to be tripled in size after 3 weeks of culture (Fig. 5 a, b)

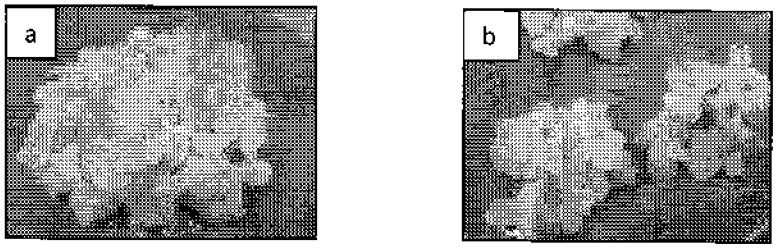

Fig. 5 a, b: Multiplication of callus derived from leaves and stem segments

2.0 Establishment of cell suspension cultures

Materials and Methods

Callus derived from leaves and stem segments were cultured in 200 ml flask containing liquid modified MS medium supplemented with 1 mg/l BAP + 0.5 mg/l Thidiazuron (TDZ). After 7 days of culture, it is vital for the suspensions to be sieved using strainer with the pore size of 1mm during the sub-culturing process. This is done for achieving uniformity and smaller sizes of cells/ fine cells (<1mm). Fine cell aggregates are preferred as these cells shows high proliferation and faster growth rate.

Results and Discussion

To establish a good and fast growing cell suspension cultures, determination of optimal inoculums density is crucial. Inoculums density of a cell culture is one of important factors that trigger the biomass accumulation as well as secondary metabolites production. It has been reported that an inoculation ratio of 1:10 (inoculums weight: medium volume) has been extensively employed in many plant cell suspension cultures (Moreno *et al.*, 1995). Size and density of inoculums of the suspension cultures greatly affected cell vitalities in further subcultures (N. A. Yusuf *et al.*, 2009)

In this study, we employed the same ratio, 5g of well dispersed and fast growing callus (Fig. 6) was inoculated in 200 ml flask containing 50 ml liquid modified MS medium supplemented with 1 mg/l BAP + 0.5 mg/l TDZ. After 7 days of culture, these suspensions were sieved with 1 mm strainer. Fine, yellowish cells i.e. < 1mm (Fig. 7) was collected and was used for further bulking up via sub-culturing. These established suspensions are sub-cultured every 14 days (middle log phase) before reaching stationary phase at 28 days. Cell aggregates (<1mm) was observed under inverted microscope (40x magnification). Cell suspension showed sign of growth and proliferation. Cells showed dense cytoplasm which is vital sign that the cells are healthy and proliferating (Fig. 8).

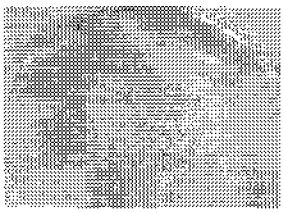

Fig. 6: Well dispersed callus

Fig. 7: Fine cell aggregates

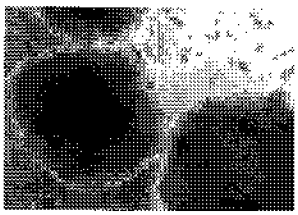

Fig. 8: Cells with dense cytoplasm

3.0 Induction and Establishment of Adventitious Roots

Materials and Methods

In vitro roots of *A.malaccensis* were cultured on modified MS medium with reduced nitrate supplemented with IAA or IBA ranging from 2 mg/l to 10 mg/l, 30 g/l sucrose with 2 g/l gelrite and pH was adjusted to 5.8. Cultures were incubated at 24°C in the dark condition. To induce multiple adventitious roots, adventitious roots induced were cut into 1.5 cm in length and inoculated into 250 ml Erlenmeyer flask containing 50 ml liquid medium of the same formulation. The cultures were maintained on shaker at the speed of 90 rpm under dark condition at 24 °C. Sub-culturing was routinely conducted at four weeks interval.

Results and Discussion

After 6 weeks of culture, adventitious roots (Fig. 9) were formed from the original *in vitro* roots. Comparatively, media supplemented with IBA were better than IAA in the formation of adventitious roots. The highest formation was observed in medium supplemented with 4 mg/l IBA. The newly developing parts of the *A. malaccensis* adventitious roots were whitish or pale yellowish and thin (Fig. 10) and later became thick and brownish as the incubation time increased (Fig. 11). Vigorous formation of multiple adventitious roots was observed in shake flasks after three months of culture (Fig. 12). With the successful and reproducible production of adventitious roots in shake flask cultures (Fig. 13), study will be conducted for the mass propagation of the *A. malaccensis* adventitious roots in air-lift bioreactor.

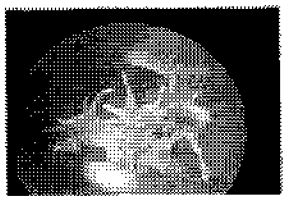

Fig. 9: Adventitious roots formed

Fig. 10: whitish and thin roots

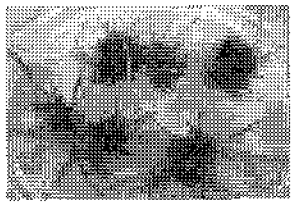

Fig. 11: brownish and thick roots

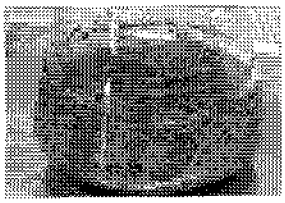

Fig. 12: Vigorous formation of adventitious roots

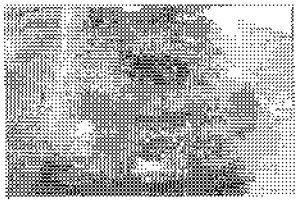

Fig. 13: Reproducible production of adventitious roots in shake flask

4.0 Conclusion

From this study, reproducible process for the induction and production of callus, cell suspensions and adventitious roots of *A. malaccensis* was successfully achieved. These established systems can be used as model systems to study the biosynthetic pathways of *gaharu* production in *Aquilaria spp.* Currently, studies on chemical elicitation by Methyl Jasmonate are being carried out using these systems for the inducement of useful secondary metabolites namely Sesquiterpenoid and 2-(2-Phenylethyl) chromones. By incorporating both the plant cell and organ systems with elicitation, metabolites can be produced under controlled and reproducible conditions. This technology also provides a better method for conservation of plant species, *Aquilaria spp* in particular.

Acknowledgement

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