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A rapid in vitro protocol for callus production in *Piper aduncum* L. propagation of East Kalimantan supplemented with gradient sucrose solutions

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Abstract. *Piper aduncum* L. is a medicinal plant which contains important active compounds such as phenyl propanoids, lignoids and flavonoids. Furthermore, it is one of larvicidal agent against mosquitoes *Aedes aegypti* L. Alternative use of in vitro callus *P. aduncum* L. are as a substitute for medicinal raw materials and becomes one way to maintain the sustainability of germ plasm utility, which is related to its difficulties in conventional cultivation. An experiment based on saline formulation of Murashige and Skoog (MS) and Wood Plant Medium (WPM) combined with different 2,4-D and kinetin concentration was evaluated. Several conditions without plant growth regulator hormones with nodule segment explants produced good multiplication process. Inoculated micro-cutting segments of nodules were grown in media with an additional 2 mg/L of 2,4-D, 2 mg/L of kinetin and 30 g sugar produced multiplication of clear shoots and callus formation and were developed after 4 weeks of planting. Additional treatment of 60, 90, 120 g sugar showed slow growth as indicated by callus formation after 6-8 weeks of planting. Treatment of 30 g sugar showed better growth than other treatments, which triggered the survival of callus growth until 12 weeks after planting. The other treatments showed cell death after callus initiation due to browning process.

1. Introduction

Piper aduncum L. belongs to family Piperaceae, a native plant from tropical regions such as South and Central America, Asia and Pasific Islands. Its leaves are used in folk medicine to treat stomach ache, trachoma and other diseases, and can be used as insect repellent. This plant is considered as shrub-like species and a natural resource for great commercial value, due to its respective secondary compounds such as dillapiol and safrole, which are the major compounds of essential oils of this plant. Piperaceae contains secondary metabolites compounds such as phenyl propanoids [1], ligninans and neolignans [2], aliphatic and aromatic amides [3], alkaloids [4] and polyketides [5].

The importance of chemical compounds isolated from Piperaceae is remarkable, for instance, the high interest of lignoids (lignans, neolignans and related chemical substances) is due to its wide range of biological activities, such as antitumor, antifungal, bactericidal, anti-PAF and anti-HIV [6], as well as the amides, with outstanding potential as insecticidal agents, molluscicides and fungicides [7].

According to the Data Base of Tropical Rain Tree, in South Mexico region, Caribbean and most of South America tropical countries described that the leaves of *P. aduncum* L. has been produced namely Matico as a traditional medicinal plant of Indian tribe. The product is infusion-form, liquid extracts, and capsules with the medicinal use as major antiseptic in discontinue bleeding, preventing infection, improving wound healing, stop vomiting, relieving dizziness, aid digestion, releasing gas from stomach, killing germs, yeast/fungi and the other benefits i.e. reduce mucus, relieve coughs and decongestants, help urinary tract and kill the virus. Matico herbal products generated from *P. aduncum* L. contains active chemical compounds including flavonoids, sequiterpenes, monoterpenes, heterocycles, phenyl propanoids, alkaloids, and benzenoids [1].

A group of chemical compounds called chromene, has been identified in leaves (essential oil) and is proven to be toxic against cancer cells and bacteria. Other chemical compounds, consist of benzenoid group and it has antibacterial and cytotoxic activities.

The problem encountered in the *P. aduncum* L. development is no reference for gathering information including their cultivation. Cultivation technology of this plant used seed as source rarely. Seed dispersal is carried out by fruit-eating animals that cause obstacles in conventional cultivation efforts. This effort requires the advanced study by using micro-propagation technique, and is expected to help the improvement of plant cultivation as a source for secondary metabolites against mosquito *Aedes aegypti* L. along with following effort for improving the *P. aduncum* L. propagation.

Plant tissue culture mainly encompasses the experimental method for growing large number of isolated cells or tissue under sterile and controlled conditions. Callus is undifferentiated and unorganized mass of plant cells. It is basically a tumor tissue which is commonly formed on wounds of differentiated tissue or organ. One method that can be used is using plant tissue culture.

In this study, we focussed on the in vitro culture of *Piper aduncum* L. propagation by measuring callus production and its secondary metabolite of rendemen yields.

2. Materials and methods

2.1. Apparatus sterilization and media preparation

Glassware and dissecting set were sterilized in autoclave at 121 ° C for 30 minutes. Media were prepared into two parts: ZPT-free media and callus growth media supplemented with ZPT (or plant growth regulator) [8]. Media for callus treatment supplemented with ZPT, 2 mg/L kinetin and 2 mg/L 2,4-D.

2.2. Explants planting

2.2.1. Nodules/plant shoot sterilization. Nodule buds were washed with detergent, soaked in fungicide and bactericidal solution for 30 minutes, washed with running water for 30 minutes. Then, this process is carried out in a laminar air flow cabinet. Plant seeds were soaked in 70% alcohol solution for 1 minute, followed in distilled water for 5 minutes, decreasing gradual concentrations of bleaching solution for 10 minutes (30%, 20% and 10%, respectively), and the seeds finally soaked in distilled water three times for each 5 minutes.

2.2.2. Shoot nodules planting. Sterile shoot nodules were planted in growth medium (MS-0) and stored in incubator room with a light intensity of ± 1,000-2,000 Lux. After 1 month incubation, these plants were used as explants source for callus formation, while the plant organs were used during study are cotyledons, tubers, stems and roots.

2.3. Callus formation

Explants were planted on media and stored in incubator room with light intensity ± 1,000-2,000 Lux and temperature 20-25 °C for 4 months. The best callus among them will be used for sucrose treatment.

2.4. Sucrose treatment

Callus was weighed 0.5 g and planted on MS medium which supplemented with increasing gradual concentrations of sucrose (30, 60, 90, 120 g, respectively). After 3 months, callus from each treatment was tested for its phytochemical contents qualitatively. Callus which contains the most secondary metabolites will be selected for further test.

3. Results and discussion

There are several factors that influence the success of tissue culture, which are media, plant growth regulator substances, explants source and environmental factors. ZPT is a non-nutrient organic compound, which is in low concentration could stimulate the plant growth in tissue culture.

P. aduncum L. callus were obtained from in vivo and in vitro explants source. Samples from both sources were initiated on media 2,4 D 2 mg/L and kinetin 2 mg/L and supplemented with gradient sucrose concentration. Result showed that previous treatments have various amount. Moreover, a trial of media supplemented with ZPT and 30 g sucrose could effectively induce *P. aduncum* L. callus.

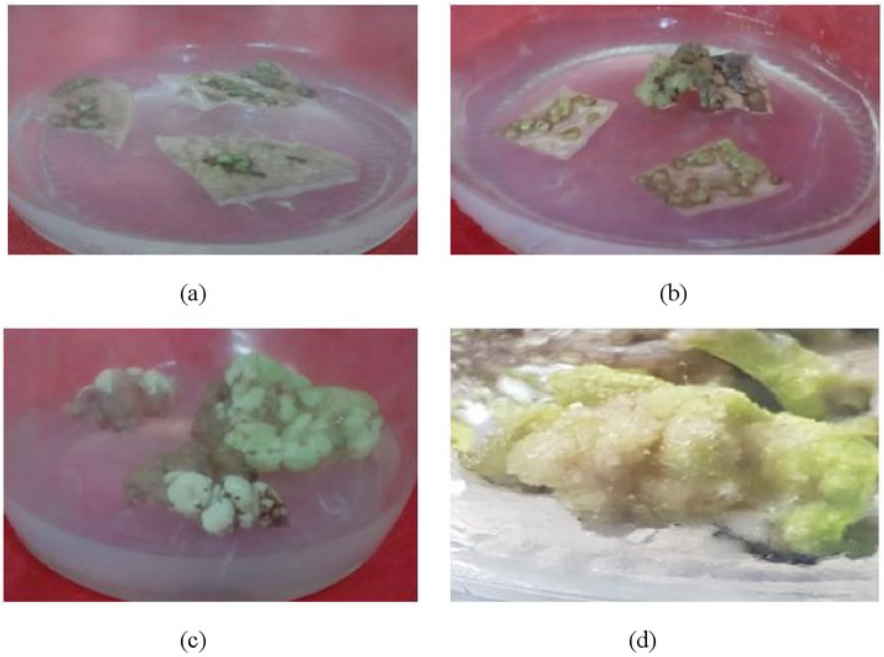

Figure 1. Optimal callus development of *P. aduncum* L. at 2 mg/L 2,4 D and Kinetin (a) 4 weeks after planting/WAP, (b) 6 WAP, (c) 10 WAP, (d) 12 WAP.

The gradual sucrose concentrations showed an increasing in sucrose utilization and hydrolyzed to glucose and fructose. Sucrose will provide better energy and carbon source in synthesizing compounds to stimulate callus growth. Sucrose also influences cell lengthening and enlargement, as seen in the results of media supplemented with 30 g sucrose, growth rates improved until the 12th week after treatment (Figure 1). Media supplemented with 60 g sucrose produced callus after 5 to 6 weeks, 90 g sucrose produced after 7 weeks and 120 g produced after 8 weeks. Hydrolyzed sucrose also causes an increase in turgor pressure [9] while this pressure increases elongation and enlargement of callus cells. Sucrose supply in the media increases the plant growth and development however the results showed

that the addition of relatively high sucrose concentration in culture media inhibit somatic cell growth [10]. This is caused by osmotic pressure which is too high, resulting in cell death according to lysis or presence of broken cell wall.

Callus color was observed either green or black. Green calli were obtained from 30 g of sucrose while black calli were obtained from 60 g to 120 g of sucrose. Different callus color showed the different responses from each treatment. Green calli possibly indicated chlorophyll presence and could develop to embryogenic callus. On the other hand, black calli was produced after browning process on explants because phenol accumulation. This chemical substance could lead to cell death or explants if not re-cultured immediately.

Finally, this paper describes a protocol for plant propagation from shoot tips and nodule segments of *P. aduncum* L. to induce the callus production. These results are important, since they allowed ex-situ conservation of genetic material and due to the seeds recalcitrance, the exchange in germ plasm could minimize the risk of pests and diseases infection. In addition, they could support a strategic domestication, which allowed their establishment in natural environment and initiated works that could lead to cell suspensions development. This previous action has aimed to induce the various secondary metabolites biosynthesis for further beneficial purposes.

4. Conclusion

This study is focussed on the in vitro culture of *P. aduncum* L. propagation by calculating callus production and secondary metabolites production grown from calli. All treatments could produce calli in various colours, either green or black colour. However, only media supplemented with 30 g sucrose produced green calli, while the others produced black calli. The green calli were more preferable, because the appearance are healthier and most likely develop embrionic cell than black calli. Data from this study will be compared with natural plant extracts for the next study in order to promote the sustainability of industrial forest, especially for *P. aduncum* L. for providing the new organic larvacidal sources.

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