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In Vitro Culture Manipulation on Pruatjan for Secondary Metabolite Production

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ABSTRAK

Manipulasi Kultur *In Vitro* pada Tanaman Purwoceng untuk Produksi Metabolit Sekunder. ***Ika Roostika, Ragapadmi Purnamaningsih, Ireng Darwati, dan Ika Mariska.*** Purwoceng (*Pimpinella pruatjan* Molk. atau *Pimpinella alpina* KDS.) adalah tanaman obat langka yang dapat dimanfaatkan sebagai bahan obat afrodisik, diuretik, dan tonik. Kultur *in vitro* tidak hanya dapat digunakan untuk konservasi dan perbanyakan tanaman, melainkan dapat juga diterapkan untuk produksi metabolit sekunder. Melalui teknik ini, produksi metabolit sekunder tidak bergantung kepada sumber tanaman di lapang. Penelitian ini dilakukan dengan tujuan untuk meningkatkan kadar stigmasterol melalui kultur *in vitro* dengan menggunakan prekursor asam mevalonat. Penelitian dibagi menjadi dua tahap, yaitu induksi kalus dan manipulasi kultur *in vitro* untuk meningkatkan kadar stigmasterol. Pada tahap induksi kalus, terdapat 16 perlakuan yang merupakan kombinasi perlakuan 2,4-D dan pikloram masing-masing pada taraf 0,5; 1,0; 1,5; dan 2,0 ppm. Untuk meningkatkan kadar stigmasterol, digunakan asam mevalonat pada taraf 0, 250, 500, dan 750 ppm dengan masa inkubasi selama 4 dan 6 minggu. Kandungan stigmasterol dianalisis menggunakan GC-MS. Hasil penelitian menunjukkan bahwa media P2 (DKW + 2,4-D 0,5 ppm + pikloram 1,0 ppm) adalah media terbaik untuk induksi kalus. Eksplan daun lebih baik daripada eksplan petiol. Hasil analisis GC-MS menunjukkan bahwa kandungan stigmasterol tertinggi (0,0356 ppm) diperoleh dari kalus dengan masa inkubasi 4 minggu pada media dengan penambahan asam mevalonat 250 ppm. Peningkatan taraf asam mevalonat tidak mampu meningkatkan kandungan stigmasterol. Kadar tersebut mirip dengan kandungan stigmasterol pada planlet dari Gunung Putri (0,0365 ppm) dan Dieng (0,0414 ppm). Dibandingkan dengan kadarnya dalam akar tanaman dari lapang, kandungan tersebut sekitar 10-100 kali lipat lebih tinggi.

Key words: Kultur *in vitro*, metabolit sekunder, *Pimpinella pruatjan* Molk.

BACKGROUND

Indonesia has a megabiodiversity of plant genetic resources, the second largest in the world after Brazil, including those of the medicinal plants. Since medicinal herb industries in the country were mostly obtained their raw materials from nature without efforts to intensively cultivate and they use the material excessively of the natural capacity, medicinal plant species are, therefore, the most eroded plants among agricultural crop species. Until l992, there were at least 30 medicinal plant species are categorized as eroded, including the commercial plant of Pruatjan (*Pimpinella pruatjan* Molk. or *Pimpinella alpina* KDS.). This plant can be used as an ingredient for diuretic, aphrodisiac, and body fit enhancers or tonics. The use of Puatjan as an aphrodisiac compound has even been patented by the University of Diponegoro, Semarang, Central Java (http://www.laksamana.net/vnews.cfm?ncat=34news_id=6397).

Based on the erosion levels, many medicinal plants in Indonesia were categorized as extinct, endangered, rare, and indeterminate (Rifai *et al.* l992). Pruatjan is categorized as one of the endangered species. Recently, Rahardjo (2003) and Syahid *et al.* (2004) reported that Pruatjan plant was currently grown by farmers only in a small area at Sekunang village, Dieng Plateau, Central Java. It is quite difficult, therefore, to fullfil enough supply of raw materials of Pruatjan plant for production of medicines. To solve this limitation, an alternative technology needs to be developed.

In vitro culture is not only applicable for plant conservations and propagations but also for secondary metabolite manipulations since this technique does not dependent on plant sources from the field. Secondary metabolites, such as terpenoid, glycoside (steroid, phenol), and alkaloid are products from plant morphogenetic processes. The *in vitro* culture offers a better way for secondary metabolite productions, especially medicinal compounds, than production from intact plants (Wetter and Constable 1991). This technique has been applied in production of secondary metabolite from several plants. Some metabolites were accumulated in cultured cells at a higher level than those in the native plants, particularly when optimization of the cultural conditions was optimized. For examples, ginsenosides of *Panax ginseng*, rosmarinic acid of *Colleus blumei*, shikonin of *Lithospermum erythrorhizon*, diosgenin of *Dioscorea*, ubiquinone-10 of *Nicotiana tabacum* were accumulated in much higher levels in cultured cells than those in the intact plants

(Misawa 1994). It is expected, therefore, that *in vitro* manipulation can also be done in the production of secondary metabolites of pruatjan.

Under *in vitro* culture, the addition of precursors or elicitor into a medium could increase the secondary metabolite contents (Ravishandar and Grewal 1991). The precursor used in the culture may dependent on structures of secondary metabolites to be produced. A pruatjan plant produces stigmasterol as the secondary metabolite. This compound is formed through the mevalonic biosynthesis pathway (Vikery and Vikery 1981). Wikinson *et al.* (1994) reported that the addition of mevalonic acid on cell suspension of celery culture increased sitosterol content, higher than control. In accordance to this, the use of mevalonic acid may also increase concentration of stigmasterol in the pruatjan culture, which is dependent on the concentration and exposure time of the precursor. Therefor, the objective of the study was to develop an *in vitro* technique for the production of stigmasterol from pruatjan through *in vitro* culture by using mevalonic acid as a precursor.

MATERIALS AND METHODS

The research was conducted at theTissue Culture Laboratory of Biology Cell and Tissue Culture Division, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development from February 2004 to December 2005. The pruatjan plant materials used in the *in vitro* cultures were collected from Gunung Putri, Bogor, West Java. The cultures were proliferated on a DKW (Driver and Kuniyaki) basal medium with additions of 1 ppm BA (benzyl-adenine), 0.2 ppm thidiazuron, and 100 ppm arginine. The cultures were subcultured every two-months and incubated at 9°C, 1000 lux, with a 12-hour photoperiod. This research was divided into two experiments, i.e., induction of callus formation and *in vitro* manipulation of the stigmasterol content.

Induction of Callus Formation

The explants used in the study were petioles and leaves of *in vitro* culture of pruatjan. These explants were cut into squares of 0.25 cm^2 and subsequently placed in the DKW basal medium. Sixteen treatments were used for callus inducing compounds consisting of combinations of 2,4-D (2,4-dichlorophenoxyacetic acid) and picloram with concentrations of 0.5, 1.0, 1.5, and 2.0 ppm, respectively. The parameters observed were initiation time of callus formation, percentage of callus formation, as well as fresh weight and dry weight of the calli.

In Vitro Manipulation to Increase Stigmasterol Content

The explants used in this study were were calli of pruatjan. The calli were maintained in the best media formulation for callus formation that obtained from the previous experiment. After one month of culturing, the calli were transferred into a fresh medium. Mevalonic acid was added to the fresh medium to increase the stigmasterol production. Concentrations of the mevalonic acid used were 0, 250, 500, and 750 ppm, respectively. The cultures were then incubated at room culture of about 20°C for 4 and 6 weeks. The parameter observed was the stigmasterol content, which was detected by the GC-MS.

The calli were harvested after certain periods of incubation time (4 and 6 weeks). Samples of approximately 1.0 g were freeze-dried overnight. One gram of dried samples were ground and soaked in a-100 ml methanol (p.a). This was then filtered by a Whatman paper no. 41 followed by silica gel (60GF254). The filtrate was then injected to the GC-MS apparatus. The plantlets (*in vitro* accessions) that were collected from Dieng, Central Java and Gunung Putri, West Java, were also analyzed by the GC-MS, to com-pare the secondary metabolite contents.

RESULT AND DISCUSSION

Induction of Callus Formation

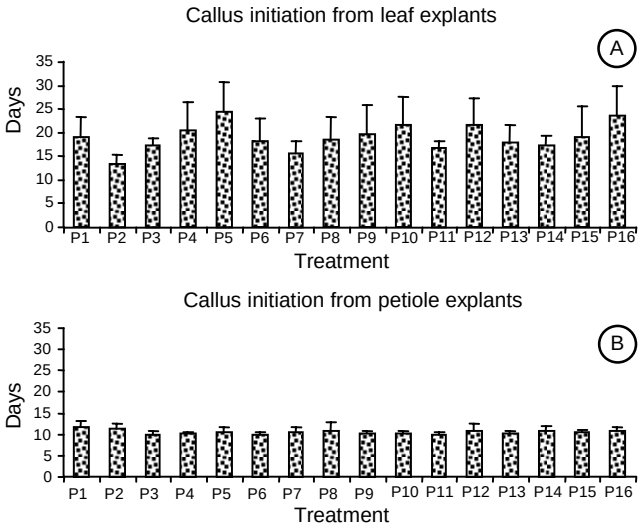
A large amount of plant materials were needed to produce secondary metabolite under *in vitro* culture. A callus is an undifferentiated cell with high level of cell division. Therefore, callus is used in the *in vitro* culture, since it can proliferate quickly and continuously. In order to obtain a large amount of calli, the application of a high activity auxin is needed, such as 2,4-D, 2,3,5-T, dicamba, and picloram. Results of the callus induction experiment indicated that callus initiations could be induced from pruatjan leaves and petioles. Generally, the growth response of the leaf was more variable than response of the petiole. Moreover, the callus initiation from petioles looked more uniform. According to Ramulu (1985), leaves and roots were possible to have polysomic cells, while according to Gunawan (1988), explants from stems, roots, and leaves commonly produced heterogenous cells. Figure 1 showed that the initiation times of calli formations from the petioles was 9 days after planting, earlier than that from the leaves, 13 days after planting. It is assumed that the petiole cells were looser than those of the leaf cells. Gunawan (1988) reported that a callus resulted from parenchyma cells showed a loose binding.

The percentage of calli formations from petioles ranged from 87.5 to 100%, which was higher than those from the leaves (62.5-93.8%) (Table 1). Growth of calli from the leaf explants, however, were faster than those from the petioles. This was indicated by the leaf explants that produced heavier fresh weight and dry weight calli (Figure 2 and 3). These figures also showed that the treatments that gave higher levels of calli fresh weights did not always produced higher level of dry weights. Among all treatments, the leaf explant that was grown on the P2 medium (2,4-D 0.5 ppm + 1.0 ppm picloram) gave the best result. This medium gave the highest percentage of calli fresh weight and dry weight. The P2 medium was therefore selected as the most suitable formulation for the induction of callus formation in the secondary metabolite production.

In Vitro Culture Manipulation to Increase Stigmasterol

Pruatjan plant produced a stigmasterol, which is used as an aphrodisiac compound. According to Taufiqurrachman and Wibowo (2005), stigmasterol might be altered into testosterone that functions in sexual activity. Therefore the stigmasterol content in *the in vitro* cultures was measured. The use of phenylalanine as a precursor in callus cultures of pruatjan has been reported (Fauzi *et al.* 2005). However, the content of secondary metabolite in the culture was lower than that in the leaves extract. In this experiment, mevalonic acid was used as a precursor in the culture.

Mevalonic acid is an intermediate compound in the biosynthesis of stigmasterol, therefore the application of this precursor is expected to increase the stigmasterol content of the pruatjan culture. Results of the trial showed that when the pruatjan calli were subcultured on the medium containing mevalonic acid, they were still growing even when the highest concentration of mevalonic acid (750 ppm) was added. However, the growth of the four weeks old cultures decreased with the increasing level of mevalonic acid contents at 500 and 750 ppm. As reported by Wikinson *et al.* (1994), the excessive mevalonic addition caused accumulation intermediate compound of xycloartenol in cells which might inhibit the biosynthesis of the following compound such as sitosterol and stigmasterol. The best growth of the culture was obtained on a medium that contain 250 ppm mevalonic acid. Unfortunately, most of the cultures were contaminated with microbes, so that it needs the other explants sources to check thesstigmasterol content from 6 weeks old cultures. The condition of the sources was rather vitrified so that the growth of the 6 week-old



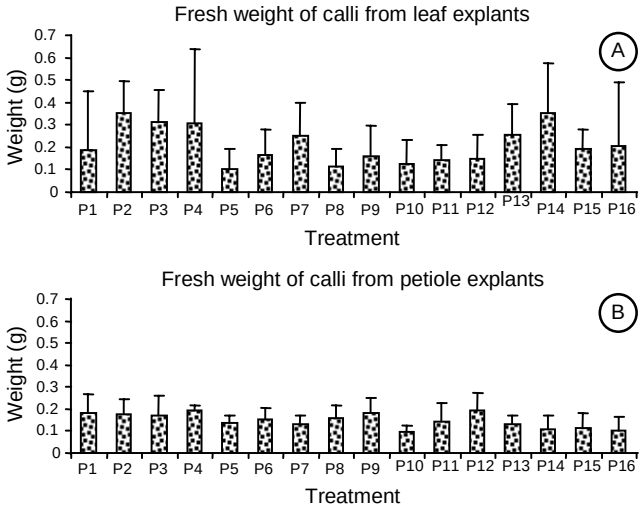
P1 = 0.5 ppm 2,4-D + 0.5 ppm picloram, P2 = 0.5 ppm 2,4-D + 1.0 ppm picloram, P3 = 0.5 ppm 2,4-D + 1.5 ppm picloram, P4 = 0.5 ppm 2,4-D + 2.0 ppm picloram, P5 = 1.0 ppm 2,4-D + 0.5 ppm picloram, P6 = 1.0 ppm 2,4-D + 1.0 ppm picloram, P7 = 1.0 ppm 2,4-D + 1.5 ppm picloram, P8 = 1.0 ppm 2,4-D + 2.0 ppm picloram, P9 = 1.5 ppm 2,4-D + 0.5 ppm picloram, P10 = 1.5 ppm 2,4-D + 1.0 ppm picloram, P11 = 1.5 ppm 2,4-D + 1.5 ppm picloram, P12 = 1.5 ppm 2,4-D + 2.0 ppm picloram, P13 = 2.0 ppm 2,4-D + 0.5 ppm picloram, P14 = 2.0 ppm 2,4-D + 1.0 ppm picloram, P15 = 2.0 ppm 2,4-D + 1.5 ppm picloram, P16 = 2.0 ppm 2,4-D + 2.0 ppm picloram.

Figure 1. Time (days) of calli initiations from leaf (A) and petiole explants (B) grown on different formulation of media.

Table 1. Effect of different media formulations on percentage of calli formations of pruatjan.

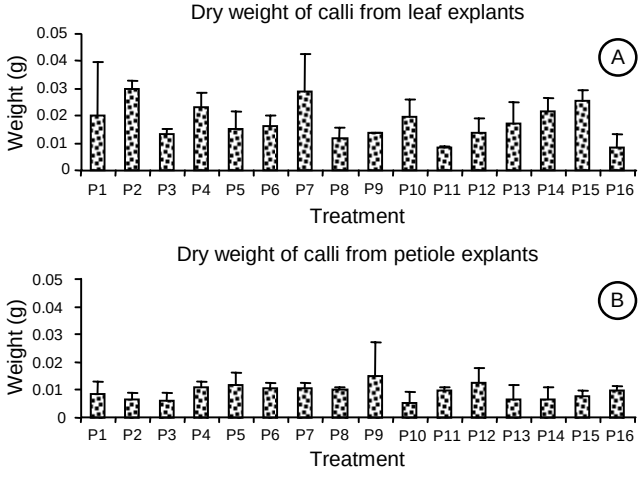
Medium formulation	Calli formations (%) from:	
	Leaf petioles	Leaves
P1	100±0	62.5±0
P2	100±0	75±35.4
P3	91.7±14.4	75±35.4
P4	100±0	81.3±8.8
P5	95.8±7.2	68.8±8.8
P6	100±0	81.3±8.8
P7	93.3±11.5	75±0
P8	95.8±7.2	93.8±8.8
P9	100±0	81.3±26.5
P10	100±0	62.5±17.7
P11	91.7±14.4	93.8±8.8
P12	100±0	87.5±17.7
P13	100±0	93.8±8.8
P14	100±0	75±35.4
P15	87.5±12.5	62.5±17.7
P16	100±0	75±35.4

P1 = 0.5 ppm 2,4-D + 0.5 ppm picloram, P2 = 0.5 ppm 2,4-D + 1.0 ppm picloram, P3 = 0.5 ppm 2,4-D + 1.5 ppm picloram, P4 = 0.5 ppm 2,4-D + 2.0 ppm picloram, P5 = 1.0 ppm 2,4-D + 0.5 ppm picloram, P6 = 1.0 ppm 2,4-D + 1.0 ppm picloram, P7 = 1.0 ppm 2,4-D + 1.5 ppm picloram, P8 = 1.0 ppm 2,4-D + 2.0 ppm picloram, P9 = 1.5 ppm 2,4-D + 0.5 ppm picloram, P10 = 1.5 ppm 2,4-D + 1.0 ppm picloram, P11 = 1.5 ppm 2,4-D + 1.5 ppm picloram, P12 = 1.5 ppm 2,4-D + 2.0 ppm picloram, P13 = 2.0 ppm 2,4-D + 0.5 ppm picloram, P14 = 2.0 ppm 2,4-D + 1.0 ppm picloram, P15 = 2.0 ppm 2,4-D + 1.5 ppm picloram, P16 = 2.0 ppm 2,4-D + 2.0 ppm picloram.



P1 = 0.5 ppm 2,4-D + 0.5 ppm picloram, P2 = 0.5 ppm 2,4-D + 1.0 ppm picloram, P3 = 0.5 ppm 2,4-D + 1.5 ppm picloram, P4 = 0.5 ppm 2,4-D + 2.0 ppm picloram, P5 = 1.0 ppm 2,4-D + 0.5 ppm picloram, P6 = 1.0 ppm 2,4-D + 1.0 ppm Picloram, P7 = 1.0 ppm 2,4-D + 1.5 ppm picloram, P8 = 1.0 ppm 2,4-D + 2.0 ppm picloram, P9 = 1.5 ppm 2,4-D + 0.5 ppm picloram, P10 = 1.5 ppm 2,4-D + 1.0 ppm picloram, P11 = 1.5 ppm 2,4-D + 1.5 ppm picloram, P12 = 1.5 ppm 2,4-D + 2.0 ppm picloram, P13 = 2.0 ppm 2,4-D + 0.5 ppm picloram, P14 = 2.0 ppm 2,4-D + 1.0 ppm picloram, P15 = 2.0 ppm 2,4-D + 1.5 ppm picloram, P16 = 2.0 ppm 2,4-D + 2.0 ppm picloram.

Figure 2. Fresh weights of calli from leaf (A) and petiole explants (B) grown on different formulation of media.



P1 = 0.5 ppm 2,4-D + 0.5 ppm picloram, P2 = 0.5 ppm 2,4-D + 1.0 ppm picloram, P3 = 0.5 ppm 2,4-D + 1.5 ppm picloram, P4 = 0.5 ppm 2,4-D + 2.0 ppm picloram, P5 = 1.0 ppm 2,4-D + 0.5 ppm picloram, P6 = 1.0 ppm 2,4-D + 1.0 ppm picloram, P7 = 1.0 ppm 2,4-D + 1.5 ppm picloram, P8 = 1.0 ppm 2,4-D + 2.0 ppm picloram, P9 = 1.5 ppm 2,4-D + 0.5 ppm picloram, P10 = 1.5 ppm 2,4-D + 1.0 ppm picloram, P11 = 1.5 ppm 2,4-D + 1.5 ppm picloram, P12 = 1.5 ppm 2,4-D + 2.0 ppm picloram, P13 = 2.0 ppm 2,4-D + 0.5 ppm picloram, P14 = 2.0 ppm 2,4-D + 1.0 ppm picloram, P15 = 2.0 ppm 2,4-D + 1.5 ppm picloram, P16 = 2.0 ppm 2,4-D + 2.0 ppm picloram.

Figure 3. Dry weight of calli from leaf (A) and petiole explants (B) grown on different media formulations.

Table 2. Stigmasterol content in pruatjan cultures on a medium containing different levels of mevalonic acid.

Medium	Culture fresh weight (g)	Culture dry weight (g)	Dry material (%)	Stigmasterol (ppm)
R4 Mev0	4.5763	0.4197	9.04	0.005919
R4 Mev250	4.1271	0.3850	9.33	0.035624
R4 Mev500	3.9665	0.1815	4.58	0.001644
R4 Mev750	3.2275	0.1524	4.72	0.013154
R6 Mev0	4.1953	0.2280	5.43	0.001754
R6 Mev250	4.6299	0.2979	6.43	0.011290
R6 Mev500	4.3495	0.2616	6.01	0.001315
R6 Mev750	2.5334	0.2180	4.66	not detected

Data in each column indicating the total amount of four replications. R = age of the cultures in weeks, and Mev = mevalonic acid in ppm.

cultures were not very good. The dry materials were only about 5% (Table 2).

The results showed that stigmasterol was produced in the callus culture of pruatjan, either on medium with or without mevalonic acid. This result suggested that stigmasterol can be produced *in vitro* from undifferentiated tissues (callus). Table 2 also shows that stigmasterol content from four weeks old calli were higher than from six weeks old calli. This level of stigmasterol content in the culture was dependent on the level of dry material of the callus. The stigmasterol content was low or undetected when the amount dry material of the culture was also low (Table 2). This result suggests that the amount of callus biomass was the most important variable in the production of pruatjan secondary metabolites (stigmasterol). A similar result was reported by Hiraoka *et al.* (2004) on cultures of *Corydalis ambigua,* when the callus fresh weight increased gradually until the end of incubation period, its dry weight increased only in the first nine days and remained constant thereafter.

The highest stigmasterol content (0.0356 ppm) was obtained from a four week-old cultures on media containing 250 ppm of mevalonic acid. This content was almost similar to the stigmaterol content of pruatjan plantlets that was collected from Gunung Putri, Bogor (0.0365 ppm) and Dieng, Central Java (0.0414 ppm). When the content was compared with that of root from a 9 month-old intact plant (Rahardjo and Darwati 2005) it was about ten to a hundred times higher.

This results proved that *in vitro* technique can be applied to produce secondary metabolite of pruatjan. However, this technique needs to be optimized by using good plant materials, other suitable media formulations, and optimum level of mevalonic acid in the media, as well as the use of other precursors. Siregar *et al.* (2005) reported that the combination of

2,4D and NAA could produce good quality of callus and induced cell suspension with high rate of growth and high level of alkaloid in *Eurycoma longifolia* cultures.

CONCLUSIONS

A secondary metabolite stigmasterol was produced from *in vitro* culture of pruatjan. The P2 (DKW + 0.5 ppm 2,4-D + 1.0 ppm picloram) was the most suitable medium for the induction of callus formation. Leaf explants was better than petiole explants for the secondary metabolite production. Results from the GC-MS analysis showed that the highest level of stigmasterol production was obtained from a four-weeks old of callus culture that was applied with 250 ppm mevalonic acid. This content was similar to that produced in plantlets collected from Gunung Putri, West Java (0.0365 ppm) and Dieng, Central Java, which was 0.0414 ppm. As compared to that produced in roots of a 9-month old intact plant, this content was about ten to a hundred times higher.

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