**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 pruataj 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 media dengan penambahan asam mevalonat 0,5; 1,0; 2,5; dan 750 ppm. Hasil penelitian menunjukkan bahwa media P2 (DKW + 2,4-D 0,5 ppm + pikloram 1,0 ppm) adalah media terbaik untuk induksi kalus. Eksplan dinaikkan sekitar 10 kali lipat lebih tinggi.

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

**BACKGROUND**

Indonesia has a megadiversity 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 excessive-ly of the natural capacity, medicinal plant species are, therefore, the most eroded plants among agricultural crop species. Until 1992, there were at least 30 medicinal plant species were categorized as eroded, including the commercial plant of Pruatjan (Pimpinella pruataj 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 Pruatjan 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. 1992). 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 fulfill 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 depend 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). Wilkinson 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. Therefore, 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 the Tissue 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 (benzyladenine), 0.2 ppm thidiazuron, and 100 ppm arginine. The cultures were subcultured every two-months and adenine), 0.2 ppm thidiazuron, and 100 ppm arginine.

RESULT AND DISCUSSION

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 cm² 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 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 compare the secondary metabolite contents.
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 dry weights did not always produce 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 Taufigur Rachman 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 Wilkinson 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 the stigmasterol content from 6 weeks old cultures. The condition of the sources was rather vitrified so that the growth of the 6 week-old 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.

**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±10      |
| 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.

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

**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            | 0.03154            |
| R6 Mev0  | 4.1953                   | 0.2280                 | 5.43            | 0.001754           |
| R6 Mev250| 4.6299                   | 0.2979                 | 6.43            | 0.01290            |
| R6 Mev500| 4.3496                   | 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.
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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