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Regeneration of Pruatjan (*Pimpinella pruatjan* Molk): Axillary Bud Proliferation and Encapsulation

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ABSTRAK

Regenerasi Tanaman Purwoceng (*Pimpinella pruatjan* Molk): Proliferasi dan Enkapsulasi Tunas Aksilar. *Ika Roostika, Ireng Darwati, dan Ika Mariska.* Purwoceng (*Pimpinella alpina* KDS atau *Pimpinella pruatjan* Molk.) merupakan tanaman obat asli Indonesia yang terancam punah. Akarnya dapat dimanfaatkan sebagai obat afrodisiak, diuretik, dan tonik. Teknik kultur *in vitro* merupakan teknologi alternatif yang dapat diterapkan untuk konservasi dan perbanyakan tanaman tersebut. Mikropropagasi telah dilakukan melalui jalur organogenesis dengan proliferasi tunas aksilar dan enkapsulasi. Penelitian dilakukan di Laboratorium Kultur Jaringan BB-Biogen, Bogor mulai tahun 2004 hingga 2005. Penelitian ini terbagi atas empat percobaan, yaitu (1) optimasi lingkungan tumbuh kultur, (2) optimasi formulasi media untuk proliferasi tunas aksilar dan enkapsulasi tunas aksilar, (3) induksi perakaran, dan (4) aklimatisasi. Kondisi lingkungan kultur yang optimum adalah di *growth chamber* dengan suhu 9°C dan intensitas cahaya 1000 lux. Formulasi media terbaik untuk proliferasi tunas aksilar adalah media DKW dengan penambahan BA 4 ppm dengan eksplan berupa tunas tanpa daun. Penggunaan arginin 100 ppm lebih baik daripada glutamin 100 ppm dan modifikasi vitamin (mioinositol 100 ppm dan thiamine-HCl 1 ppm). Pada media yang sama, pertumbuhan tunas aksilar terenkapsulasi juga paling baik dan tunas tersebut dapat menembus kapsul alginat setelah 4 minggu dalam periode *in vitro* (85%). Penggunaan NAA 1,0 ppm menginduksi perakaran paling cepat (40 hari) dengan persentase perakaran paling tinggi (100%). Vermikulit bertekstur kasar paling baik untuk aklimatisasi tunas aksilar terenkapsulasi sedangkan arang sekam paling baik untuk aklimatisasi planlet.

Kata kunci: Organogenesis, enkapsulasi, proliferasi tunas aksilar, *Pimpinella pruatjan* Molk.

INTRODUCTION

Pruatjan (*Pimpinella alpina* KDS or *Pimpinella pruatjan* Molk.) with local names purwoceng, suripandak abang, gebangan depok, and antanan gunung, is a medicinal plant with a high economic value. Its roots can be used as aphrodisiac, diuretic and tonic. Preclinical test indicated that the root extract of pruatjan has androgenic and estrogenic activities (Caropeboka 1980; Kosin 1992; Taufiqqurrachman 1999; Juniarto 2004). Many traditional herb industries (*jamu*) are looking for this material for commercial use.

Pruatjan is an Indonesian indigenous plant which is growing endemically in mountainous area, such as Dieng Plateau and Lawu Mountain in Central Java, Pangrango and Galunggung Mountain in West Java, as well as Tengger and Iyang Highland in East Java (Heyne 1987). Recently, the population of this plant has become rare because of large genetic erosion. Most of traditional herb industries harvested the materials directly from its natural habitats without any replanting activities. Since the commonly used part is root, its harvest will then be destructive to the whole plant.

Based on the genetic erosion level, Pruatjan is categorized as endangered (Rivai *et al.* 1992). Therefore, it is included in the Appendix I of CITES (Convention of International Trading in Endangered Species) so it is highly protected. Its natural habitat has extinct due to the forest damage. Rahardjo (2003) and Syahid *et al.* (2004) reported that the plant was only grown in small scale by farmer at Sekunang village in Dieng Plateau. In order to avoid extinction of this plant, conservation needs to be done immediately. The conservation may be done both through *in situ* and *ex situ*. Since *in situ* conservation is quite impossible to be applied due to no more available conservation forest, therefore *ex situ* conservation becomes important, such as *in vitro* conservation.

Regeneration method is a very important step that has to be managed before plant conservation. Regeneration can be done through organogenesis and somatic embryogenesis. The regeneration through organogenesis by axillary bud proliferation has been applied. However, the cultures were difficult to form root and easy to form callus and senescence, so that frequent subculturing is needed (Mariska *et al.* 1990; 1995; Rahayu and Sunarlim 2002). It is predicted that optimum culture conditions and media formulations are needed to optimize the regeneration process. Thus, the objectives of the research were to optimize culture conditions, medium formulation, and acclimatization medium for propagation of pruatjan through organogenesis, particularly by the axillary bud proliferation.

MATERIALS AND METHODS

The research was conducted in the Tissue Culture Laboratory of the Indonesian Center for Agriculture Biotechnology and Genetic Resources Research and Development (ICABIOGRAD) during the year of 2004-2005. The plant materials were *in vitro* cultures of pruatjan which were collected from Gunung Putri, Cipanas-West Java. The research activities were divided into 4 experiments, i.e. (1) optimization of culture condition, (2) optimization of medium formulation, (3) induction of root formation, and (4) plant acclimatization.

Optimization of Culture Conditions

Single axillary buds with leaves were used as explants. The explants were planted on the DKW medium containing 2 ppm benzyladenine (BA). The room conditions for culture incubations were room culture at 800 lux, 27°C and growth chamber with different kinds of light intensity (800, 1000, or 1500 lux) 9°C. The observed variables were number of leaves, total number of leaves, number of wilted leaves, and visual performance of the cultures.

Optimization of Medium Formulation

Axillary bud proliferation

This experiment used axillary buds with or without leaf as the explants. The treatments were different levels of BA concentration (1, 2, 3, 4, and 5 ppm) on the DKW basal medium. The cultures were incubated in growth chamber at 1000 lux and 9°C. The observed variables were number of shoots, total number of leaves, number of wilted leaves, and visual performance of the cultures. The best concentration of BA treatments was then combined with amino acid (100 ppm arginine or glutamine) or vitamin modification containing of 100 ppm myo-inositol, 1 ppm thiamine-HCl (Charoensub *et al.* 1999) to optimize regeneration of the cultures.

Encapsulation

In this experiment, axillary buds (±0.5 cm in length) were used as the explants. The axillary buds were encapsulated by 2.5% Na-alginate containing BA that has been dipped in a 100 mM $CaCl_2$ solution for 15 minutes. The amount of BA was given at the same level as that in the previous experiment. The observed variables were percentage of survived shoots, percentage of emerged shoots, percentage of root formations, and number of leaves.

Root Induction

Root induction of axillary buds (from axillary bud proliferation) was conducted *in vitro,* while root induction of the encapsulated axillary buds was conducted *ex vitro* before acclimatization. The DKW basal media containing indole butyric acid (IBA) or naphthalene acetic acid (NAA) at the level of 0.5, 1.0, and 1.5 ppm were used for root induction. The observed variables were percentage of root formations, time initiation of root formations, and visual performance of the cultures.

Acclimatization

The plant acclimatizations were conducted in two steps. In the first step, encapsulated axillary buds were used while in the second step plantlets were used as the materials. Before the acclimatization, the encapsulated axillary buds were dipped in a 30 ppm IBA solution for 5 hours. The encapsulated axillary buds were then planted on different media i.e. soil, coarse vermiculite, fine vermiculite, and cocopeat. The best medium was applied to acclimatize plantlets and compared with rice husk charcoal medium. The media were autoclaved. Plants were incubated in a growth chamber at 1000 lux and 9°C. The observed variables were percentage of plant survivals.

RESULT AND DISCUSSION

The optimal condition is very important for supporting the growth of plant culture. Previous research reported that pruatjan was very difficult to be manipulated *in vitro* because of its high level of senescence and callusing, low level of shoot multiplication, and also its difficulty for root formation (Mariska *et al.* 1990). It is assumed that the temperature was not appropriate for the plant growth since they were incubated in a culture room 25-27°C. To optimize the culture growth, lowering the temperature may support the growth of the culture since pruatjan was endemic to mountainous area with low temperatures.

Results of the experiment showed that at the same light intensity i.e. 800 lux at the culture room and 800 lux in the growth chamber, the lower temperature provided the better growth of cultures which indicated by higher number of shoots and total number of leaves than those in higher temperature. However, at light intensity 800 lux, leaf senescence (wilting) could not be avoided. The optimal growth of the cultures was obtained from growth chamber with 1000 lux light intensity. Under this condition, the number of shoots and total number of leaves were highest than those from other treatments, and without any senescence. At

the same time, the leaf blade condition was also normal (Table 1).

The most important variable for axillary bud proliferation is the number of shoots since the buds are utilize as propagules for propagation. Result of the study also indicated that generally the shoot multiplications were low on all the tested media. The growth of cultures was also dependent on the level of BA and the type of explants. The best growth was obtained on media containing 4 ppm BA using shoots without leaves as the explants (Table 2 and Figure 1). The plant regeneration could be improved by modifying the media. The shoot multiplications could increase up to 5.0 axillary buds/explant, when the medium was supplemented with 100 ppm arginine (Table 3). Mariska *et al.* (1995) reported that pruatjan cultures provided 4.25 axillary buds/explant at 14 weeks of *in vitro* culture period, however leaf senescence was still present. Similar results were reported by Miftakhurohmah *et al.* (2005), axillary bud proliferations provided low level of shoot multiplications (5.0 axillary buds/explant).

The encapsulation of *in vitro*-derived axillary bud has been employed in recent years to develop synthetic seeds (Lestari *et al*. 2000; Noviati and Roostika, 2005). The alginate coat protects the micropropagules and thus has a practical application in plant exchanges and conservations. The provision of alginate coat as an artificial endosperm to provide nutrients is essential for development of encapsulated micropropagules (Danso and Ford-Lloyd 2003). Therefore, in the encapsulation experiment, different medium formulations were tested. The BA was given at the same level as that in the previous experiments to ensure that the non-capsulated and the encapsulated axillary buds needed the same level of BA.

Results of the encapsulation experiment showed that the encapsulated axillary buds survived and grew on all the tested media. They even could emerge from the capsules one month after planting (Figure 2). The

Table 1. The effect of culture conditions (temperatures and light intensities) on growth of pruatjan cultures, 4 weeks after planting on DKW basal media containing of 2 ppm BA.

Treatment	Number of shoots	Total number of leaves	Number of wilting leaves	Visual performance of culture
Culture room, 800 lux, ±27°C	1.0±0	2.25±0.96	0.25±0.50	Leaf blade is wide
Growth chamber, 800 lux, 9°C	1.25±0.50	2.50±1.0	0.75±0.96	Leaf blade is wide
Growth chamber, 1000 lux, 9°C	1.67±1.15	5.0±3.46	0	Leaf blade is normal
Growth chamber, 1500 lux, 9°C	1.40±0.55	3.40±2.07	0	Shoot meristem is brown

Table 2. The effect of BA levels in the DKW basal media and type of explant on growth pruatjan cultures, 10 weeks after planting.

BA (ppm)	Number of shoots		Total number of leaves		Number of wilting leaves	
	L	WL	L	WL	L	WL
1	1.25±0.50	1.0±0	2.75±0.50	3.0±0.82	0	0
2	1.0±0	1.50±1.0	2.5±0.58	3.5±0.58	0	0
3	2.0±0.82	1.25±0.50	6.0±3.37	3.25±1.71	0	0
4	1.25±0.50	2.75±1.71	3.50±0.58	8.0±5.03	0.25±0.50	0
5	1.75±0.96	2.0±0.82	6.0±3.56	3.75±1.50	0.25±0.50	0

L = with leaves, WL = without leaves.

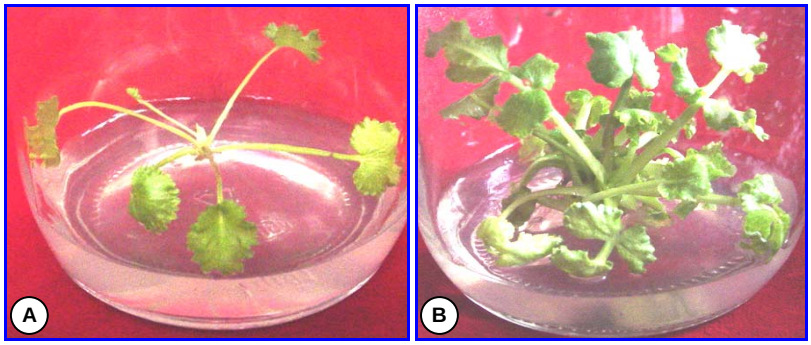

Figure 1. Axillary bud proliferation of pruatjan. A = lower level of shoot multiplication and B = higher level of shoot multiplication.

growths of the encapsulated axillary buds were varied among the treatments (Table 4). Besides supporting shoot growth, the treatments also supporting the root growth. However, the high shoot growth was accompanied by the low root growth. It seems that they did not need *in vitro* root inductions, thus the root induction was enough to be conducted *ex vitro* prior to the acclimatization.

The level of 4 ppm BA provided the highest level of culture growth as indicated by the highest level of shoot emergence percentage (85%). This result was similar to result on a previous experiment of axillary bud proliferation. It was shown that the 4 ppm BA was the optimum level for plant regeneration through organogenesis particularly by using axillary buds as the explants. From this result, however, indicated that organogenesis by axillary buds proliferation was not effective for pruatjan propagation, because the shoot multiplication level was very low. Other regeneration techniques are therefore needed to be applied, such as somatic embryogenesis.

Results of the root induction experiment showed that root formation of axillary buds could be induced by both NAA and IBA. However, the application of NAA was better than IBA since it induced root formations up to 100%. The best medium for the root induction was DKW basal medium containing 1.0 ppm NAA. The roots were visually thicker than those on other treatments (Table 5). The same result was also reported by Syahid *et al.* (2004) when pruatjan cultures were planted on MS basal medium containing NAA. According to Moore (1979), NAA is more active, more persistent, and easier to be translocated in plant tissues.

Acclimatization is one of the most important steps for a successful micropropagation. Results of the encapsulated axillary buds acclimatization showed that they could survive for three weeks after planting on both coarse and fine vermiculite media. After three weeks, they could not survive longer. After five weeks of planting, the percentage of survival was decreasing. On soil and cocopeat media, their media growths were contaminated with fungi (Table 6 and Figure 3), because humidity of the materials was high, thus it induced contamination. Moreover, the cocopeat might contain organic and inorganic compounds that are preferred by the fungi. Result of the plantlet acclimatization showed that rice husk charcoal medium (60%)

Figure 2. The growth of encapsulated axillary buds of pruatjan. A = the original condition of the encapsulated buds, B = most of the axillary buds were still protected with the capsules, and C = most of the axillary buds had emerged from their capsules.

Table 3. The effect of medium modification on growth of pruatjan cultures, 16 weeks after planting.

Treatment	Number of shoot	Total number of leaf	Number of wilt leaf
Arginine 100 ppm	5.0±3.46	11.50±5.80	0.75±0.50
Glutamine 100 ppm	2.0±1.0	8.86±6.56	0
Modified medium	2.75±1.50	11.25±7.27	0

Modified medium contained 100 ppm myo-inositol and 1 ppm thiamine-HCl.

Table 4. The effect of different BA levels on growth of encapsulated axillary buds of pruatjan, 4 weeks after planting.

BA (ppm)	Percentage of shoot (%)	Percentage of emerged shoot (%)	Percentage of root (%)	Average of leaf number
1	80	55	25	1.35
2	90	80	15	2.35
3	100	65	10	1.65
4	100	85	10	2.30
5	100	80	0	2.50

Table 5. The effect of different auxins and concentrations on root induction of pruatjan cultures.

Auxin (ppm)	Percentage of rooting (%)	Time of root initiation (days)	The thickness of root
NAA 0.5	100	49±10.7	+
NAA 1.0	100	38.5±11	+++
NAA 1.5	100	55±0	++
IBA 0.5	25	34±0	-
IBA 1.0	75	48±12.1	-
IBA 1.5	33	55±0	-

+ = more or thicker roots, - = less or thiner roots.

Table 6. The effect of different acclimatization media on growth of encapsulated axillary buds of pruatjan.

Medium	Percentage of survival material (%)		Note
	3 weeks	5 weeks	
Soil	60	0	Contaminated
Cocopeat	70	0	Contaminated
Coarse vermiculite	100	30	Clean
Fine vermiculite	100	20	Clean

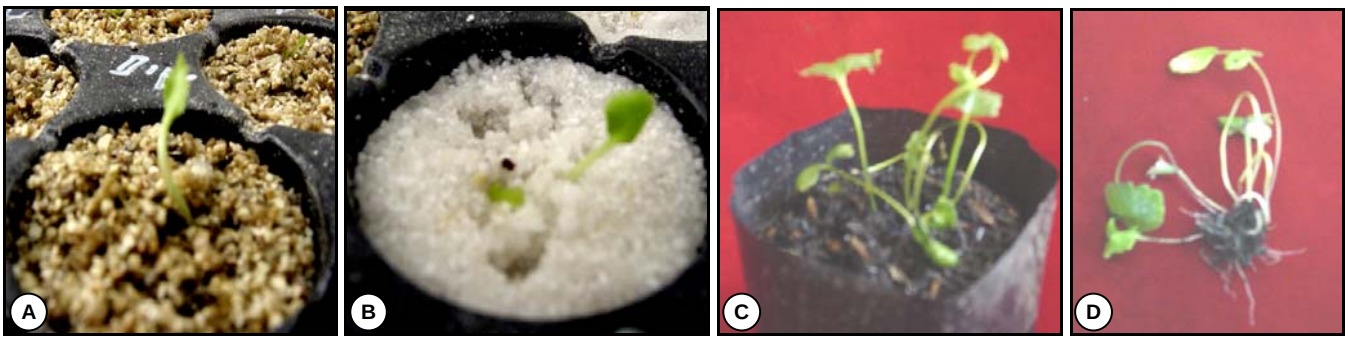

Figure 3. The visual performances of acclimatized materials of pruatjan. A = encapsulated axillary buds on coarse vermiculite, B = encapsulated axillary buds on fine vermiculite, C and D = seedling on a rice husk charcoal medium.

was better than coarse vermiculite (0%) until two months after planting. Two months after planting, the plantlets were still survived and the roots grew well (Figure 3).

CONCLUSION

Pruatjan could be regenerated by organogenesis through axillary bud proliferation and encapsulation. Optimize culture condition has been obtained. The best incubation condition for regeneration was in a growth chamber with 9ºC temperature and 1000 lux light intensity. The best medium for axillary bud proliferation was DKW basal medium containing 4 ppm BA, using a single bud without leaf as the explant. The growth of encapsulated axillary buds was also highest and the most of buds emerged from the capsules four weeks after the *in vitro* culture period (85%) on the same medium. The use of 1.0 ppm NAA in the DKW basal medium provided the earliest time of root induction (40 days) and the highest percentage of root formation (100%). Vermiculite, either coarse or fine texture, was better than rice husk charcoal to acclimatize encapsulated axillary buds, but rice husk charcoal was better than vermiculite to acclimatize plantlets.

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