

## REGENERATION OF *Pimpinella pruatjan* THROUGH SOMATIC EMBRYOGENESIS

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### ABSTRACT

Pruatjan (*Pimpinella pruatjan* Molk.) is an Indonesian endangered plant which has various medicinal properties such as aphrodisiac, diuretic, and tonic. The plant is commonly harvested from its natural habitat, therefore it becomes endangered. Regeneration of pruatjan through organogenesis has been studied, but its shoot multiplication was very low (5 shoots per explant). The study aimed to investigate the best regeneration technique of pruatjan through somatic embryogenesis. This research was conducted at the tissue culture laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development in 2004-2005. Callus formation of pruatjan was induced from the petioles and leaves in Driver and Kuniyaki's (DKW) based medium containing 2,4-D combined with picloram at the level of 0.5, 1.0, 1.5, and 1.5 ppm. Embryogenic calli were then transferred into embryo development medium in two ways. First, they were directly transferred into media containing IBA/NAA at the level of 0.5, 1.0, and 1.5 ppm. Second, they were indirectly transferred into media containing 2.0 ppm 2,4-D and 0.3% casein hydrolysate prior to the IBA/NAA media. Parameters evaluated were fresh weight, dry weight, time initiation of embryogenic callus formation, and total number of embryos. The result showed that calli of pruatjan were successfully induced from the petioles and leaves. The best calli were induced from the leaves in the DKW medium containing 2.0 ppm 2,4-D and 0.5 ppm picloram. Embryo development of the calli was best if they were first grown in the media containing 2.0 ppm 2,4-D and 0.3% casein hydrolysate then transferred to the IBA/NAA media. The total number of somatic embryos was counted up to 103 on the medium containing 1.5 ppm IBA. This study indicated that pruatjan somatic embryogenesis regeneration required three different media, i.e. for callus induction, development and maturation, and for germination.

[Keywords: *Pimpinella pruatjan*, somatic embryogenesis, plant regeneration]

### INTRODUCTION

Pruatjan (*Pimpinella pruatjan* Molk.) is a very important medicinal plant that can be used as aphrodisiac, diuretic, and tonic materials. The use of pruatjan as aphrodisiac has been patented by the University of Diponegoro (www.laksmana.net 2004). Traditional

herb industries requires large quantities of pruatjan herb, but its supply is limited because the plant is only grown in limited areas in Dieng Plateau, Central Java. Most of the herb was directly harvested from its natural resources, therefore the plant becomes endangered (Rivai *et al.* 1992). Rahardjo (2003) and Syahid *et al.* (2005) reported that pruatjan was only grown by limited farmers in Sekunang village in Dieng Plateau.

Pruatjan was successfully cultivated outside its natural habitat of Dieng Plateau, but the main problem is scarcity of seeds or seedling materials (Rahardjo 2003) because of its harvesting method practices by digging up the whole plant, since the roots are mainly used for medicinal purposes. Therefore, it is important to find alternative technique for propagating the plant, such as using *in vitro* culture technique.

The propagation of pruatjan through organogenesis especially by axillary bud proliferation has been studied (Herawati 1991; Mariska *et al.* 1995; Miftakhu-rohmah *et al.* 2005). However, this technique is not effective because it produced limited numbers of shoot multiplication (5 shoots per explant). To increase the shoot multiplication and root formation, it is interesting to evaluate other regeneration methods such as somatic embryogenesis. Unfortunately, no report was found on pruatjan.

Somatic embryogenesis is a regeneration process of somatic cells that develop by division to form complete embryos analogous zygotic embryos. The bipolar structure of the somatic embryo contains both shoot and root meristems. As the embryos develop, they progress into the distinct structural steps of the globular, heart, torpedo, cotyledonary, and mature stages (Phillips *et al.* 1995). Somatic embryogenesis technique is a promising method for micropropagation, even though it is more suitable for automation and scale-up than organogenesis since it does not require the cutting of plantlets into segments and transferring the segments onto new media during proliferation (Kurata 1995 *in* Ibaraki and Kurata 2001). This technique is probably suitable for mass pro-

pagating of pruatjan as well as for long-term conservation such as cryopreservation of calli and plant improvement through somaclonal variation and genetic engineering. Principally, in the somatic embryogenesis a single cell can be induced to become a somatic embryo. The establishment of somatic embryogenesis has been reported on several plants such as cassava (Danso and Ford-Lloyd 2002), ginseng (Choi *et al.* 2000; Zhou and Brown 2005), sugarcane (Nieves *et al.* 2003), and camellia (Janeiro *et al.* 1997).

The main steps in somatic embryogenesis are callus induction and regeneration. The explants should be planted on suitable medium to induce embryogenic callus formation, then the calli must be transferred to a regeneration medium to promote germination of the somatic embryos. To support the embryogenic callus formation, it needs some inductive compound. There are certain organic and inorganic chemicals required for callus induction and embryogenesis development, such as plant growth regulator of 2,4-D (Wang 1987; Choi *et al.* 1999), dicamba (Osuna and Barrow 2004), and picloram (Groll *et al.* 2001). However, the combination of growth regulators sometimes is good to induce callus formation in somatic embryogenesis regeneration as reported by Przetakiewicz *et al.* (2003) on Triticale and Yusuf *et al.* (2001) on *Piper colubrinum*. This study aimed to find a suitable regeneration technique of pruatjan through somatic embryogenesis.

## MATERIALS AND METHODS

The research was conducted at the tissue culture laboratory, Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development from February 2004 to December 2005. Plant materials used were *in vitro* cultures of pruatjan originally collected from Gunung Putri, Cipanas, West Java (1,545 m above sea level). The cultures have been maintained *in vitro* for about 8 months, with frequent subcultures so that they are always juvenile. The *in vitro* shoots of pruatjan were multiplied on the Driver and Kuniyaki's basal medium (Driver and Kuniyuki 1984) or DKW medium containing 4 ppm benzyl adenine (BA) and 100 ppm arginine (Roostika *et al.* 2006). The cultures were incubated at 20°C with 800-1000 lux light for 16-hour photoperiodicity. The cultures were routinely subcultured every 2 months. The study consisted of two main experiments, i.e. embryogenic callus induction and somatic embryo development, followed with acclimatization.

## Embryogenic Callus Induction

Petioles and leaves of the *in vitro* cultures of pruatjan were cut into 0.25 cm<sup>2</sup> size. They were then put on the DKW basal medium supplemented with combination of 2,4-D (2,4-dichlorophenoxyacetic acid) and picloram at concentrations of 0.5, 1.0, 1.5, and 2.0 ppm, respectively. The number of replications was 5-8 explants each treatment.

The explants were incubated at dark condition with temperature about 20°C. Observation of visual performances was conducted every week. The parameters observed were fresh weight and dry weight of 1-month old calli. The data of fresh weight were taken from two groups of experiment while the data of dry weight were taken from one group of experiment since drying process is destructive to the cells so that it is impossible to regenerate the calli after drying.

Data were analyzed using ASREML software (Gilmour *et al.* 2001) and presented as a graph, and the best linear unbiased predictions (BLUPs) were calculated for every random terms. They were used without putting back the general means. Therefore the data was presented as deviation from mean. The models that used for analyzing were presented in Table 1. In this case, the media was fixed while the source (leaves and petioles) was random since they were taken randomly from population of shoots.

## Somatic Embryo Development

Two-month-old calli (about 0.6 g weight) from the previous experiment were subjected to two different protocols of embryo development. First, they were directly transferred to Petri dishes containing DKW based media supplemented with indole butyric acid (IBA) or naphthalene acetic acid (NAA) at different levels, i.e. 0.5, 1.0, and 1.5 ppm, respectively. Second, the calli were transferred to Petri dishes containing DKW based medium supplemented with 2 ppm 2,4-D and 0.3% casein hydrolysate prior to the IBA/NAA media as described before. Because of limited numbers of calli available, for the experiment the treatment was not replicated. Cultures were incubated at 20°C and 800-1000 lux light for 16-hour photoperiodicity in a culture room.

Parameters observed were time initiation of the embryogenic callus formation which was observed every week and the total number of embryos which was accounted from 2-months-old cultures after subculturing. The data were presented as the mean and standard of deviation.

**Table 1. Models for ASREML analysis.**

Observation	Model
Dry weight	$y_{jkl} = \mu + \alpha_j^* + \beta_k + (\beta\gamma)_{kl} + \varepsilon_{jkl}$
Fresh weight	$y_{ijkl} = \mu + \Theta_i^* + (\alpha \Theta)_{ij}^* + \beta_k + (\beta\gamma)_{kl} + (\Theta\beta)_{ik} + (\Theta\gamma)_{il} + (\Theta\beta\gamma)_{ikl} + \varepsilon_{ijkl}$

Note: \* set as fixed effect

where:

$y_{jkl}/y_{ijkl}$	= observation data
$\mu$	= overall mean
$\Theta_i$	= effect of group i
$\alpha_j$	= effect of replication j
$(\alpha \Theta)_{ij}$	= effect of replication j within group i
$\beta_k$	= effect of explants source k
$(\beta\gamma)_{kl}$	= effect of interaction between explants source k and media l
$(\Theta\beta)_{ik}$	= effect of interaction between group i and explants source k
$(\Theta\gamma)_{il}$	= effect of interaction between group i and media l
$(\Theta\beta\gamma)_{ikl}$	= effect of interaction between group i, explants source k, and media l
$\varepsilon_{jkl}/\varepsilon_{ijkl}$	= error

### Plantlets Acclimatization

A protocol for acclimatization of pruatjan plantlets followed the method of Roostika *et al.* (2006) with slight modification. Plantlets were planted on an autoclaved rice husk charcoal medium in polybags, incubated in culture room for 1 month, and transferred on chambers containing soil and compost (1:1). To keep high humidity condition, the chambers were covered by cling wrap plastic. After 2 months in that acclimatization period, the covers were gradually opened until the chambers were uncovered.

## RESULTS AND DISCUSSION

### Embryogenic Callus Induction

Calli of pruatjan were successfully induced from the *in vitro* leaves and petioles explants. The calli in general contained water more than 80% of their fresh weights. Fresh weight of calli induced from the leaves was higher (0.10-0.36 g) than those from the petioles (0.08-0.18 g). Similarly, dry weights of calli from the leaves were higher (0.01-0.03 g) than those from the petioles (0.005-0.018 g). This might be due to the starting sizes of the explants, where the surface of calli originated from the leaves were bigger than those of the petioles. The calli developed from the leaves also had more sub-epidermal cells in mesophyll layers which responded more to nutrition presented in

the callus induction media used. The petioles probably contained more monophenolic compounds such as lignin which inhibit cell division so that growth of the calli from the petioles was slower than those from the leaves.

Those different responses might also be caused by the capability of cells of the explants. Pedroso and Pais (1995) showed that embryogenic capability of *Camellia japonica* explant is a region-specific under culture condition even in the same explants such as leaf segments. From this report it was known that the defined regions of midrib were respond to somatic embryogenesis. Since it was better to use leaves as the explants for callus induction so that this research would only be concerned on leaves explants.

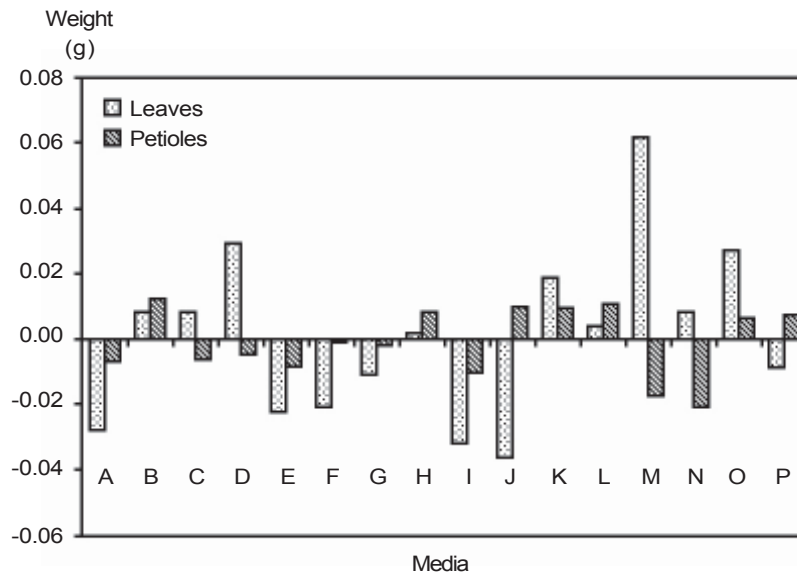
The best media for fresh weight of the calli was DKW based medium containing 2.0 ppm 2,4-D and 0.5 ppm picloram (Fig. 1) which provided low level of dry weight (Fig. 2). It seems that DKW based medium containing 2.0 ppm 2,4-D and 0.5 ppm picloram resulted calli with high water content. The high water content indicated the high content of sucrose, organic acid, and also amino acid which are essential for supporting the development of the embryogenic calli to form somatic embryos.

Similar phenomenon was found by Hiraoka *et al.* (2004) in the *Corydalis ambigua* cultures treated with cytokinin where the fresh weight of somatic embryos increased and the dry weight decreased, associated with the cytokinin activities which absorbed much water. On the callus regeneration of pruatjan it seems that 2,4-D and picloram at 2.0 ppm and 0.5 ppm, respectively, caused cell expansion and extension of cell division activated by cytokinin. Thus, exogenous auxins present in DKW based medium (2,4-D and picloram) induced endogenous cytokinin biosynthesis resulting in high level of fresh weight.

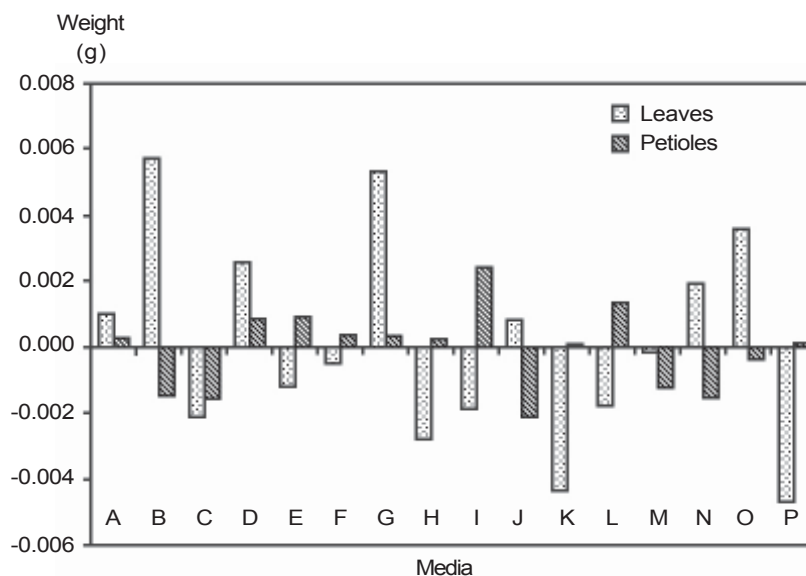
### Somatic Embryo Development

Although the calli produced in the DKW based medium containing 2.0 ppm 2,4-D and 0.5 ppm picloram have highest fresh weight, but after 4 weeks of culturing, the calli did not develop and mature, i.e did not contain bipolar cells (shoot polar and root polar). This means that the calli should be transferred into different growth medium containing IBA or NAA as suggested by Ibaraki and Kurata (2001).

Transferring the calli into the medium containing 2.0 ppm 2,4-D and 0.3% casein hydrolysate resulted shorter time for initiation of embryogenic calli (only 1 week), higher level of the total number of somatic embryos (up to 103 embryos), and also better perfor-



**Fig. 1.** Fresh weight of 1 month calli of pruatjan from several medium formulations. A = 2,4-D0.5 P0.5, B = 2,4-D0.5 P1.0, C = 2,4-D0.5 P1.5, D = 2,4-D0.5 P2.0, E = 2,4-D1.0 P0.5, F = 2,4-D1.0 P1.0, G = 2,4-D1.0 P1.5, H = 2,4-D1.0 P2.0, I = 2,4-D1.5 P0.5, J = 2,4-D1.5 P1.0, K = 2,4-D1.5 P1.5, L = 2,4-D1.5 P2.0, M = 2,4-D2.0 P0.5, N = 2,4-D2.0 P1.0, O = 2,4-D2.0 P1.5, P = 2,4-D2.0 P2.0, (2,4-D = dichlorophenoxyacetic acid, P = picloram, and all levels were in ppm).



**Fig. 2.** Dry weight of 1 month callis of pruatjan from several medium formulations. A = 2,4-D0.5 P0.5, B = 2,4-D0.5 P1.0, C = 2,4-D0.5 P1.5, D = 2,4-D0.5 P2.0, E = 2,4-D1.0 P0.5, F = 2,4-D1.0 P1.0, G = 2,4-D1.0 P1.5, H = 2,4-D1.0 P2.0, I = 2,4-D1.5 P0.5, J = 2,4-D1.5 P1.0, K = 2,4-D1.5 P1.5, L = 2,4-D1.5 P2.0, M = 2,4-D2.0 P0.5, N = 2,4-D2.0 P1.0, O = 2,4-D2.0 P1.5, P = 2,4-D2.0 P2.0, (2,4-D = dichlorophenoxyacetic acid, P = picloram, and all levels were in ppm).

mance of embryogenic calli which were yellowish friable. It seems that the indirectly transferring into medium containing 2.0 ppm 2,4-D and 0.3% casein hydrolysate prior to IBA/NAA media was better than the directly transferring to the IBA/NAA media. It indicated that the use of media containing auxin and

casein hydrolysate could shorten the maturation. As reported by Gunawan (1992), casein hydrolysate is a kind of amino acid that acts as organic-N source which can be absorbed by plant tissue easier than anorganic-N. According to George and Sherington (1993), the addition of amino acid on auxin containing



media can increase development of embryogenic callus because amino acid in chloroplast plays the role as precursor on formation of nucleic acid and other cellular process. The use of casein hydrolysate has succeeded in embryogenesis regeneration of sugarcane (Ho and Vasil 1983). It was reported that organized structures resembling the early stages of embryogenic calli were formed on medium containing 2,4-D but these did not develop beyond the globular stage. However, the addition of casein hydrolysate resulted in the formation of embryogenic calli.

After maturation, somatic embryos developed further to germinate. Embryo germination was indicated by the elongation of shoot structure and subsequent root initiation that may take place on the same media. However, sometimes root formation emerged before shoot elongation. The development of somatic embryos of pruatjan was illustrated in Figure 3. It seems that the embryogenic cells were bipolar (Fig. 3a-c). When they were mature, they could develop normally become plantlets which contained both shoot and root (Fig. 3g).

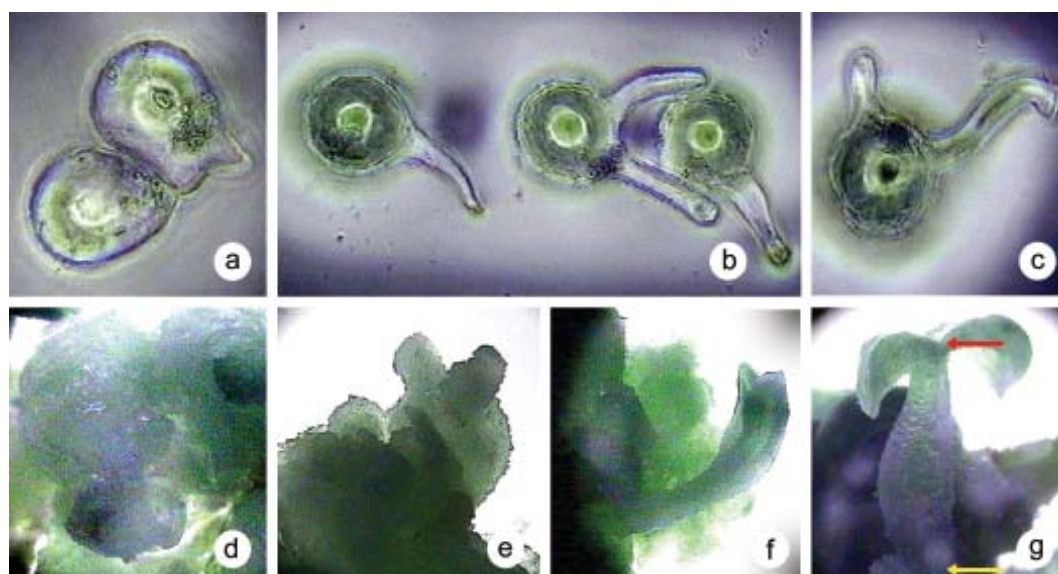
The number of mature somatic embryos was affected by the use of IBA and NAA. The number of somatic embryos was higher when the level of auxin (IBA/NAA) was higher. The highest number of somatic embryos was achieved up to 103 that obtained from 1.5 ppm IBA (Table 2). Visual observation showed that the somatic embryos from IBA were developed better than those from NAA since the

formation of shoot and root from IBA treatment was balance (Fig. 4a). The root from NAA treatment was dominant and thicker (Fig. 4b). The mature somatic embryos could germinate on the same media containing IBA or NAA. However, the growth of plantlets from IBA treatment (Fig. 4c) was faster than those from NAA treatment (Fig. 4d). Conclusively, in the case of pruatjan somatic embryogenesis regeneration, there were three different media for callus induction (DKW + 2.0 ppm 2,4-D + 0.5 ppm picloram), initiation of embryo development and maturation (DKW + 2.0 ppm 2,4-D + 0.3% casein hydrolysate), and also for germination (DKW + 1.5 ppm IBA).

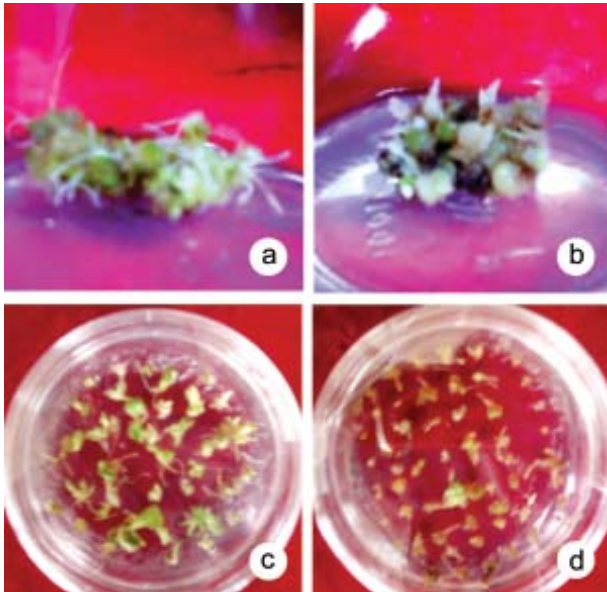
**Table 2. Regeneration of pruatjan cultures through somatic embryogenesis, 2 months after subculture.**

Treatment (ppm)	Time initiation of embryogenic calli (weeks)		Number of somatic embryos	
	Step 1	Step 2	Step 1	Step 2
IBA 0.5	4	1	7	17
1.0	5	1	0	51
1.5	5	1	4	103
NAA 0.5	5	1	1	33
1.0	5	1	3	45
1.5	5	1	15	contaminated

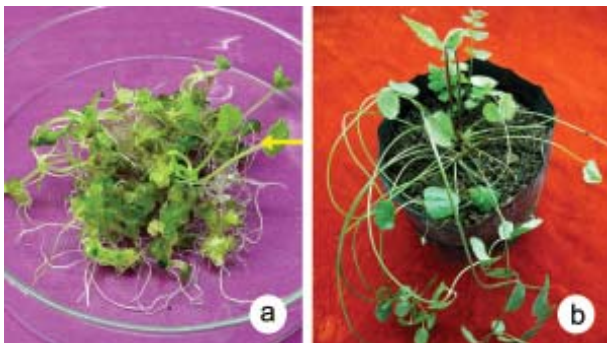
Note: Step 1: calli grown on DKW + 2.0 ppm 2,4-D + 0.5 ppm picloram, Step 2: calli from the step 1 was then subcultured on DKW + 2.0 ppm 2,4-D + 3 g l<sup>-1</sup> casein hydrolysate.



**Fig. 3.** The development of somatic embryos of pruatjan; a-c = different stage of embriogenic cells, d = globular, e = early cotyledonary, f = late cotyledonary, and g = full germination. Red arrow means apical shoot meristem, while yellow arrow means root meristem.



**Fig. 4.** The different growth stage of pruatjan somatic embryos; a = somatic embryos from media containing IBA, b = somatic embryos from media containing NAA, c = plantlets from media containing IBA, and d = plantlets from media containing NAA.



**Fig. 5.** Performance of developed seedlings; a = plantlet that was ready to be acclimated (shown by arrow) and b = 8-months old acclimatized seedling.

### Plantlet Acclimatization

Following acclimatization of the plantlets for 2-months period using the technique developed by Roostika *et al.* (2006), about 60% of the seedlings survived in the chambers which was partially covered with plastic wrap. However, after the chamber was uncovered, most of the seedlings died. This indicated that the seedlings required humid condition of their growth. Further study is required to examine degree of humidity suitable for acclimatization of the seedlings.

As mentioned before, propagation through organogenesis resulted low level of shoot multiplication,

Thus, compared to the regeneration through organogenesis by axillaries bud proliferation, somatic embryogenesis is more effective to be applied for micropropagation of pruatjan since this technique provides much more seedlings (about 200 times) because they are developed from callus (resulted from cut leaves), a mass of cells that each cell is potential to develop to be individual somatic embryo. It was concluded that somatic embryogenesis regeneration is the best technique to be applied for mass propagation of pruatjan. Further experiments should be focussed on improving acclimatization and transplanting methods to the field, to obtain higher level of survival seedlings.

### CONCLUSION

Regeneration of pruatjan can be applied through somatic embryogenesis. Leaves were better used as the explants than petioles. In the case of pruatjan somatic embryogenesis regeneration, there were three different media which were for callus induction (DKW + 2.0 ppm 2,4-D + 0.5 ppm picloram), initiation of embryo development and maturation (DKW + 2.0 ppm 2,4-D + 0.3% casein hydrolysate), and also for germination (DKW + 1.5 ppm IBA). The total number of somatic embryos was up to 103 that obtained from 1.5 ppm IBA. This technique is more effective to be applied for mass propagation of pruatjan since it may provide a plenty of seedlings.

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