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In Vitro Culture of Pineapple by Organogenesis and Somatic Embryogenesis: Its Utilization and Prospect

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ABSTRAK

Kultur *In Vitro* Nanas secara Organogenesis dan Embriogenesis Somatik: Pamanfaatan dan Peluangnya. *Ika Roostika T. dan Ika Mariska*. Sistem regenerasi pada kultur *in vitro* dapat dilakukan melalui dua jalur, yaitu jalur organogenesis dan embriogenesis. Teknik organogenesis dan embriogenesis pada tanaman nanas telah banyak diteliti, bahkan penelitian tentang perhitungan ekonominya juga sudah pernah dilakukan. Penerapan organogenesis dan embriogenesis tergantung pada tujuan yang dikehendaki. Kedua teknik tersebut dapat dimanfaatkan untuk berbagai kebutuhan seperti perbanyakan bibit, konservasi plasma nutfah, perbaikan tanaman melalui variasi somaklonal ataupun manipulasi genetik, hingga pemisahan tanaman khimera, serta pemeliharaan tanaman mutan yang menguntungkan. Prospek penerapan teknik kultur *in vitro* tanaman nanas di Indonesia khususnya di daerah Subang cukup bagus terutama untuk mengatasi permasalahan perbanyakan nanas Si Madu yang kemungkinan merupakan pertumbuhan sel mutan dari tanaman khimera. Masalah yang dihadapi dalam perbanyakan vegetatif nanas Si Madu secara konvensional adalah timbulnya keragaman sehingga sifat pohon induknya tidak dapat dipertahankan. Melalui jalur embrio-genesis, karakteristik nanas Si Madu diharapkan dapat dipertahankan. Dalam hal ini tanaman (planlet) yang dihasilkan dari jalur embriogenesis berasal dari satu sel sehingga terjadinya khimera dapat dihindari. Tanaman-tanaman hasil embriogenesis dapat diseleksi di tingkat lapang atau dapat dideteksi dengan menggunakan markah isozim dan RAPD. Tanaman yang mempunyai karakteristik nanas Si Madu dapat digunakan sebagai sumber bahan perbanyakan secara konvensional atau sebagai sumber eksplan untuk diperbanyak kembali secara kultur *in vitro.*

Kata kunci: Organogenesis, embriogenesis, nanas (*Ananas comosus*)

Pineapple [*Ananas comosus* (L.) Merr.] is a fruit crop native to southern Brazil and Paraguay. This crop is a tropical or near-tropical plant that grow in region where the temperature remains warm. This plant is suitable under Indonesian condition, such as in southern Sumatra and Java. Commonly, pineapple fruits are consumed as canned fruit, fresh fruit, juice, and jam. The demand of pineapple in the country is so high that the cultivation need to be done intensively. Intensive cultivation needs availability of sufficient planting material. Therepore, proper techniques need to be studied for mass production of the pineapple planting propagation.

Conventionally, pineapples are propagated by new vegetative growth. There are four general types of pineapple propagation, i.e. (1) use of slips arising from the stalk below the fruit, (2) suckers origin-ated from leaf axils or leaves, (3) crowns of the fruits, and (4) ratoons that come out of the underground part of the stems. The need of large number of planting material such as those for commercial or industrial use is difficult to be achieved by conventional techniques, particular-ly when uniform planting material are needed. Thus, alternative tech-nologies for production of uniform planting material in a large number need to be studied. One of the technologies that can be used for this purpose is the *in vitro* technique.

The use *in vitro* technique has two advantages. Besides it can be used to produce large number and uniform pineapple planting material in a relatively short period of time (Firoozabady and Gutterson, 2003), and can also be used to improve plant performances. Sripaoraya *et al.* (2003) reported that since culti-var improvement requires at least five years by sexual hybridization and selection, tissue culture based tech-nologies provide a crucial adjunction not only to conventional breeding but also for the propagation and genetic improvement. Since, pineapple is a monocotyledonous crop, which is self-incompatible and highly heterozygous. Genetic engineering is, important in pineapple improvement. Plant rege-neration is, therefore, a prerequisite for genetic engineering of pineapple (Firooza-bady and Moy, 2004). Evans *et al.* (1981) also mentioned that efficient protocols for plant regeneration need to be implemen-ted prior to application of cellular genetic techniques in crop improve-ment.

The *in vitro* technique that can be applied for this purpose is regeneration of pineapple culture by organogenesis and embryogenesis. Embryogenesis is more interesting than organogenesis, because this technique can be applied in production of large number of planting material in relatively short period of time. Moreover, embryogenesis can be used for plant improvement and genetic manipulation. The probability of success in production of transgenic plants is, however, higher than production of plants by organogenesis since one somatic embryo comes from one cell.

ORGANOGENESIS OF PINEAPPLE

Organogenesis is a process to form and to develop shoots from

meristematic tissue (Pardal, 2002). In plant propagation, production of high level of shoot number is desired. The shoot multiplication may be achieved by axil and terminal bud proliferation. This can be done *in vitro* using a suitable solid or liquid medium containing either cytokinin or a combination of cytokinin and auxin.

Currently, a rapid and reliable micropropagation method has been developed for *in vitro* shoot and proliferation of pineapple. The tech-nique included preparation of crown tip explants from mature fruits and culturing them on a Mura-shige-Skog (MS) medium. Multiple shoots obtained from the crown tip explants were then grown on an MS medium supplemented with differ-ent combinations and concentra-tions of benzyladenine (BA), kinetin (Kn), naphthalene acetic acid (NAA), and indole butyric acid (IBA). A maximum number of multiple axillary shoot proliferation occurred on MS basal medium containing 1.0 mg/l BA and 0.1 mg/l NAA. The *in vitro* proliferated shoots produced maximum number of roots on the MS medium supple-mented with 0.2 mg/l IBA and 0.2 mg/l NAA (Rahman *et al.*, 2001). A similar result was obtained by Firoozabady and Gutterson (2003). They used a combination of 1.5 mg/l BA and 0.5 mg/l NAA in the medium and produced the highest rate of shoot multiplication, about three to four fold monthly. They also reported that liquid media was better than the solid medium for this purpose (Table 1). Sripaoraya *et al.* (2003), however, reported that adventitious shoots were produced from the leaf bases when the ex-plants were cultured on an MS me-dium containing 0.5 mg/l (2.26 μM) 2.4-dichloro-phenoxy-acetic acid (2.4-D) and 2.0 mg/l (8.87 μM) BA (Sripaoraya, 2003).

In 2004, Firoozabady and Moy (2004) reported that they have developed two techniques for organogenesis of pineapple, i.e. direct and indirect organogenesis. The first techniques involved direct regeneration of plant shoots from plant bases, without callus formation. Alternatively, an indirect technique was done by first producing nodular globular structures followed shoot regeneration. The highest level of regeneration by direct organogen-esis was obtained on an MS me-dium containing 27 μM NAA and 1 μM BA, with a 99% regeneration percentage (Table 2), while that by indirect organogenesis, on obtained from the same medium, was only 86% regeneration percentage (Table 3). Pictorial steps of the direct and indirect organogenesis processes are shown in Figure 1. Who believed that these regener-ation systems were applicable for regeneration of any pineapple cultivars. The fact that only the leaf bases are the only pineapple leaf portion responsive to the regeneration process may implied that the leaf bases are located in the vicinities of axillary meristems that con-tain meristematic regions or pos-sess newly developed tissue with rapid dividing cells, amenable for morphogenesis in tissue culture. Firoozabady and Gutterson (2003), however, stated that beside the leaf

Table 1. Multiplication of two pineapple lines from Indonesia in solid and liquid B1.5N.5 media (MS medium +1.5 mg/l BA + 0.5 mg/l NAA)

Pineapple line	Multiplication medium	Initial no. of shoots	Final no. of shoots	Duration of culture (months)	Monthly MR[b]
4.05	Solid	22	7,478b	7	1.7->4.0
	Liquid	23	14,908a	7	1.7->4.8
4.07	Solid	27	5,608b	6	1.7->4.5
	Liquid	28	14,760a	6	1.6->4.6

[a]Values in a column followed by a different letter are significantly different at P>0.01; [b]MR (multiplication rate) was initially 1.6-1.7 month, but it increased to 4-4.6 over time

Source: Firoozabady and Gutterson (2003)

Table 2. Pineapple shoots regeneration frequencies on seven different media

Medium	No. of explants regenerated/total	Regeneration (%)[x]
N27B1	594/743	80ab
N27B1	164/166	99a
B4	151/239	63bc
B13	209/337	62c
N5B13	73/103	71abc
N1B13	183/265	69bc
N1B22	74/105	70abc

[x]Values in a column followed by different letters are significantly different at P>0.05; B = benzyladenine (μM); N = naftalenacetic acid (μM)

Source: Firoozabady and Moy (2004)

base, longitudinal part of the leaf was another responsive tissue for regeneration. The percentage of explants producing shoot and the average number of shoots per crown produced from the longitudinal section was higher than those from the leaf base (Table 4).

EMBRYOGENESIS

Micropropagation is very efficient for propagation of genetic planting material obtained from plant breeders. This is particularly so for ligneous plant species which are allogamous, where this "elite plants" cannot be propagated through seeds or even by the classical horticulture. Somatic embryogenesis, especially using cell suspensions, offers a powerful tool for propagation of pineapple planting material.

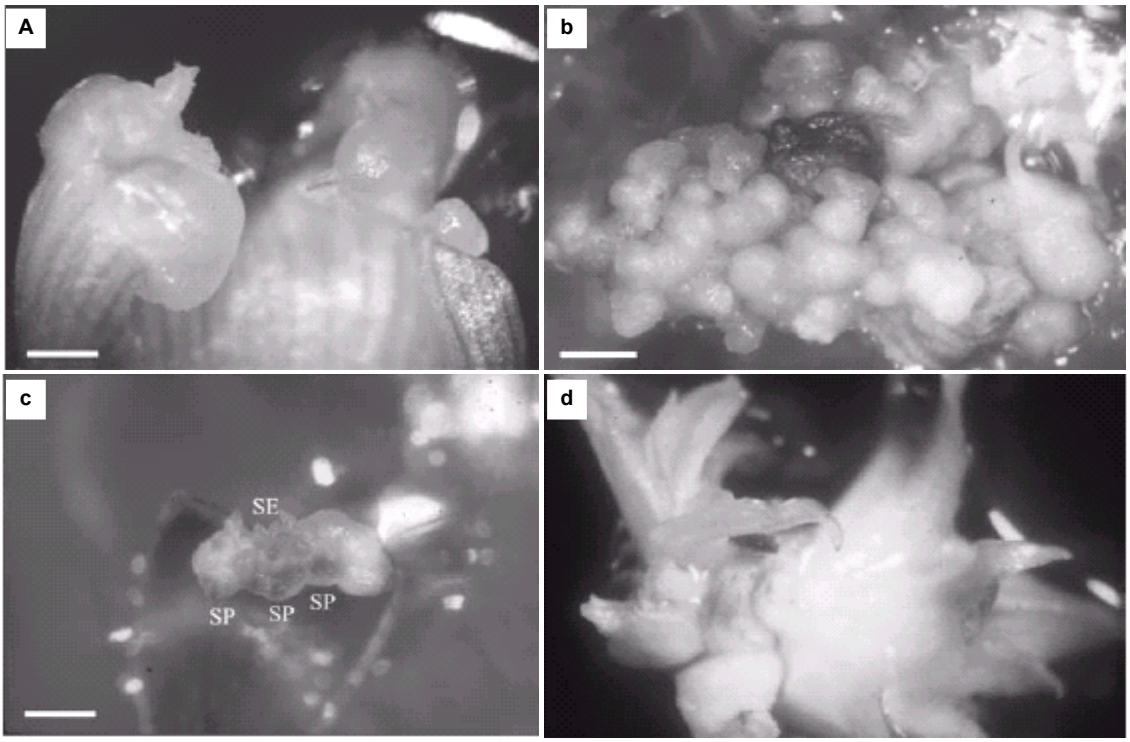
Somatic embryogenesis is a regeneration process of somatic cells that are developed by cell division to form complete embryos which are analogous to zygotic embryos. The bipolar structure of the somatic embryo contains both shoot and root meristems. As the embryos grow, their structures are

Table 3. Regeneration of shoot and nodular globular structures (NGS) from leaf bases cultured on seven different media

Medium	No. of crowns (no. of explants)	Shoot regeneration (%)	No. of shoots per crown	NGS (%)	No response (%)
N27B1	24 (1204)	86a	82a	0c	9
B4	5 (231)	47b	45b	17b	25
N16B4	5 (207)	51b	21b	27b[y]	24
T5I.5	6 (258)	50b	4c	85a	15
TI[z]	6 (252)	51b	5c	82a	16
N27B1[x]	6 (255)	47b	56b	0b	25
B4	4 (176)	82a	74a	7a	11

[a]Values in a column followed by different letters are significantly different at P>0.05; [x]On N27B1 medium, the remaining explants produced undifferentiated calluses; [y]The NGS on N16B4 medium were calluosy, some what unorganized and undesirable; [z]On TI medium, 21% of the explants produced somatic embryos directly without callus interphase; B = benzyl adenine (μM); N = naphthalene acetic acid (μM); T = thidiazuron (μM); I = indole butyric acid (μM)

Source: Firoozabady and Moy (2004)



a = nodular globular structures (NGS) production on a leaf base cultured on T5I.5 medium; b = NGS multiplication on T5I.5 medium; c = leaf bases plated on T2I2.5 medium produced primordial shoots (SP) and somatic embryos (SE); d = shoot regeneration from leaf bases carrying multiple shoot primordia; bars in: A and B = 1 mm, C = 3 mm, and D = 2 mm

Figure 1. Organogenesis in pineapple

Source: Firoozabady and Moy (2004)

Table 4. Production of pineapple shoots (buds) on four different media

Medium[a]	Source of explants	Explants producing shoots (%)	Average no. of shoots per crown[b]
N5B.2	Longitudinal section	100	110a
N5B.2	Leaf base	86	78a
N3B1	Leaf base	51	45b
B1	Leaf base	47	41b

[a]Each medium was MS medium containing different concentrations of BA and NAA as described in the materials and methods. The number following each letter on the name of medium is concentration of the respective hormone in milligrams per liter; [b]Numbers of shoots per crown followed by different letters are significantly different at P>0.01

Source: Firoozabady and Gutterson (2003)

developing into different phases, i.e. globular, heart, torpedo, cotyledonary, and mature stages (Phillips *et al.*, 1995).

Direct somatic embryogenesis is the formation of somatic embryos or embryogenic tissue directly from an explant without the formation of an intermediate callus phase (Finer, 1995).

There are reports on methods for induction of plant embryogenesis including that for plant regeneration (Daquinta *et al.,* 1996; Sripaoraya *et al.*, 2003; Firoozabady and Moy, 2004). Plant growth regulators are always used for induction of embryogenesis; the most commonly plant growth regulators are 2,4-D, dicamba, and picloram. The explants that have successfully been used to produce embryogenic tissues of pineapple were the leaf bases and core section. It was also reported that, in pineapple embryogenesis, among the leaf sections (tip, middle, and base) tested, only leaf bases were responsive and only when under light conditions. Other leaf sections were generally turned brown and eventually died. When leaf base explants used in the embryogenesis were smaller than 3 mm x 3 mm, they usually died. On the other hand, when the explants sizes were larger than 7 mm x 7 mm, they expanded but did not developed into organized or differentiated tissues (Soneji *et al.* 2002a; Firoozabady and Moy, 2004).

According to Firoozabady and Moy (2004), there were five types of embryogenic tissues in the embryogenic system, i.e., non-proliferative somatic embryos, callus-derived embryogenic tissues, chunky non-dispersible embryogenic tissues, fri-able embryogenic tissue, and highly friable embryogenic cell clusters. Indirect somatic embryogenesis occurred when high concentration of auxins (picloram, dicamba, and 2.4-D) were used in the medium. This phenomenon confoirmed the statement of Evans *et al.* (1981) that generally a high concentration of auxin and low concentration of cytokinin promotes abundant cell proliferation with the formation of callus. In the direct somatic embryogenesis (Figure 2A, B), somatic embryo formation on the explants without the callus interphase was obtained when a combination of thidiazuron and indole butyric acid hormones were tested. These embryos did not proliferated, however they developed and produced shoots directly. Embryogenic tissues (ETs) are undispersible organized structures (3-6 mm) that are produced by proliferation of immature somatic embryos. Simply, ET (Figure 2C) is a cluster of immature somatic embryos that are fused together. Friable embryogenic tis-sues (FETs; Figure 2D) are dispers-ible tissue containing individual globular embryos (0.5-1 mm) in a matrix of potentionally embryo-genic cells. Embryogenic cell clus-ters (ECCs; Figure 2E) are highly friable tissue containing cell clus-ters (20-500 + small cells, each cell is about 5-10 μM in diameter).

Soon after mature somatic embryos were obtained, they must be cultured further to form shoots. Therefore, they must be transferred immediately to the regeneration medium. Firoozabady and Moy (2004) reported that the MS basal medium was significantly better than the White medium for embryo development and shoot elongation (Table 5). The low level of embryo development and shoot elongation on the White medium may be due to its salt contents. The White medium contained lower salts than the MS medium and it was ammonium-free (Evans *et al.,* 1981). Addition of some organic compounds to the White medium did not improve the growth performance of the em-bryos (treatment no. 5). The MSB4 medium was preferred for shoot development, since it enable the embryos to produce significantly more shoots (treatment no. 1 vs. 2). The MSB1 medium was preferred for shoot elongation and growth, since it significantly enhanced the production of more shoots that were ready for rooting (treatment no. 3 vs. 4 or 5). The hormone-free MS medium (MSO) also produced a higher number of large shoots than the hormone-free White medium (WO) (Treatment no. 6 vs. 7) (Firoozabady and Moy, 2004).

UTILIZATION OF THE TECHNIQUE

Management of *in vitro* technique in pineapple might be utilized for several different purposes. As mentioned earlier, *in vitro* tech-nique can be used for pineapple plant propagation. The direct and indirect organogenesis techniques are the best choices to be applied in pineapple propagation, since the presence of genetic variability in the plant can be avoided as much as possible. Furthermore, occurrence of somaclonal variation also very much dependent on the technique. Feuser *et al.* (2003) reported that the use of temporary immersion system resulted in a lower frequency of somaclonal variation than the stationary system (1.9% versus 3.9%). The cost-effective of *in vitro* propagation methods even have been accounted by Firoozabady and Gutterson (2003). They propagated the embryo using three kinds of bioreactors, i.e., air lift bioreactor, rotating bioreactor, and periodic immersion bioreactor (PIB). Among the bioreactors, PIB was the best propagation system. This bioreactor had the capacity to produce 6,000-8,000 shoots from two initial shoots in less than 6 months. A schematic illustration of the PIB and a picture of healthy shoots produced in a PIB system are shown in Figure 3. Another bioreactor system called temporary immersion system (TIS) had been developed by Escalona *et al.* (1999). This tool, which was better than using the solid and liquid media, can be used to increase the multiplication rate, as well as fresh and dry weight of the shoots.

Table 5. Effect of MS and white (W) media on pineapple embryo and shoot development

Number[x]	Medium	No. of clusters	N. of shoots[y]	No. shoots >3 cm[y]
1	MSB4	70	145a	0
2	WB4	75	55b	0
3	MSB1	28	165a	41a
4	WB1	29	105a	10b
5	WMB1[z]	28	90a	6b
6	MSO	80	149a	39a
7	WO	79	113a	12b

[x]Treatment nos. 1 and 2 = embryogenic tissues were plated on the media,10 pieces per plate); treatment nos. 3, 4, and 5 = tiny shoot clusters (0.5-1 cm) were plated on the media, 5 to 8 clusters per plate; and treatment nos. 6 and 7 = small shoot clusters (1-2 cm) were plated on the media, 5 to 8 clusters per plate. Number of shoots were counted 6 wk after plating; [y]Values in a column followed by different letters are significantly different at P >0.01; [z]Medium WMB1 contained organic compounds, 100 mg/l each of glutamine, arginine, asparagine and 600 mg/l of casein hydrolysate

Source: Firoozabady and Moy (2004)

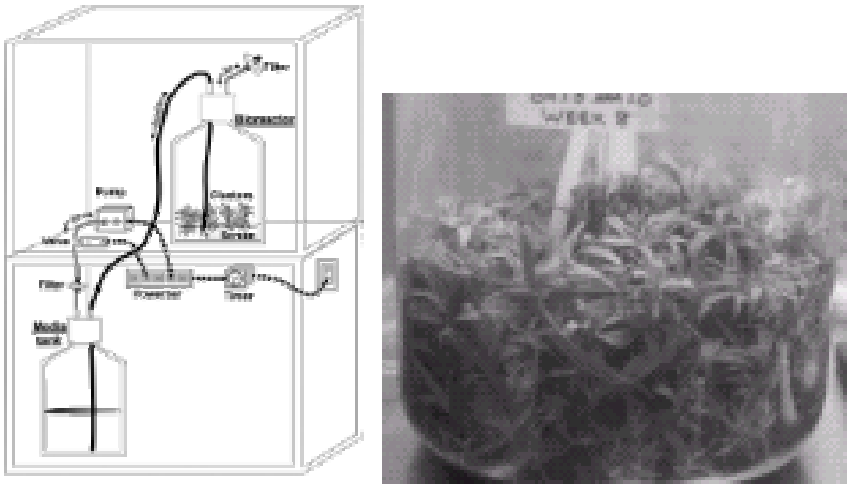
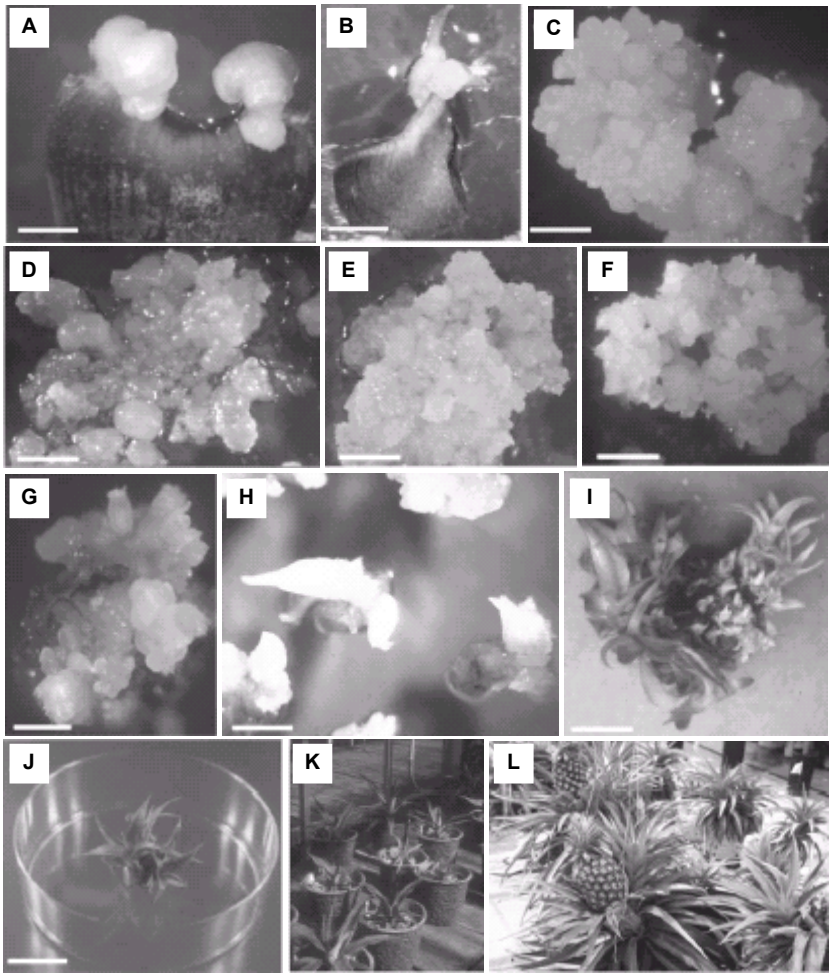


Figure 3. A schematic illustration of PIB (A) and performances of healthy pineapple shoots produced in a PIB (B)
Source: Firoozabady and Moy (2004)



A and B = direct somatic embryogenesis in leaf bases at the globular stage (A) or mature cotyledonary stage (B); C = embryogenic tissue (ET) developed after 6 wk; D = friable embryogenic tissue (FET) developed by culture of longitudinal sections; E = embryogenic cell clusters developed after FETs, cultured for 8 wk; F = embryo maturation 2 wk after ETs plating; G = embryo maturation after FETs were plated. Note the differences in efficiency of mature embryo formation; H = different types of mature embryos ready for shoot development; I = clusters of shoots and buds produced from a tiny piece of ET; J = a cluster of developed shoots from ET ready for propagation or rooting; K = plants after transfer to soil; L = mature normal plants produced in an greenhouse in Oakland, CA; bars in: A, D, E = 1 mm; B, C, G, H = 2 mm; F = 3 mm; I = 5 mm; J = 20 mm

Figure 2. Somatic embryogenesis and plant regeneration in pineapple
Source: Firoozabady and Moy (2004)

Another application of organogenesis is that for producing artificial seed. The artificial seed may act like true seed for propagation. In this term, embryogenesis may also be applied. Soneji *et al.* (2002b) reported that the synthetic seeds of pineapple shoot by using 3% sodium alginate were successfully established in the soil.

Furthermore the conservation of plant material may be conducted by using the explant from both organogenesis and embryogenesis depended on the purpose either to maintain certain plant genetic or to maintain some genetic variability. The conservation of pineapple apices by cryopreservation has been reported by Gonzales-Arnao *et al*. (1998). In this term, the applica-tion of vitrification technique with PVS2 solution was better than the encapsulation-dehydration technique.

For the purpose of plant improvement, the direct and indirect somatic embryogenesis is the best choice to be used. In this term, genetic variability is desired to obtain certain plant with some traits as we want. Since the existence of chimerical is not desired, then embryogenesis becomes a good option to be used for purpose of genetic manipulation. As we know, the organ that obtained from or-

ganogenesis might be supported from several cells that possible have different kind of genetic. Then one individual plant from organo-genesis may have chimerical in some region too.

FUTURE PROSPECT

A large pineapple plantation is available in Subang, West Java. The famous pineapple cultivar grown in this area is Si Madu. This name has been given to this cultivar for its fruit taste which is very sweet like honey (*Madu*). According to Soedibyo (1992), this cultivar produced fruits with an average diameter that meet an international standard cateory. National demand for pine-apple has been increasing from year to year, but our domestic production is still low and unable to fulfil the national consumption. Indonesia is still have to import this crop from other countries (Hadiati *et al.*, 2003)

Although pineapple is propa-gated vegetatively, a wide range of variability in characteristics of pine-apple plants occurred such as that in cultivar Si Madu. This variability is caused by mutation and influence of extreme environmental condi-tions (Hadiati *et al.*, 2003). There-fore, the characteristics of a pine-apple clones are not always the same at those of their mother plants.

In Subang district, the local pineapple cultivar Si Madu occu-pied only about 5% of the area, although farmers in the area propa-gated this crop vegetatively. One reason to explain this condition is the presence of mutation in the plant that caused the plants be-come chimerical. Probably, Si Madu was also a mutant. Therefore, a technology that can separate chi-mera and maintain the mutant is needed.

In the case of chimerical cells in pineapple, the application of em-bryogenesis technique might be able to separate the chimerical cells from the non-chimerical one, since one plant may develop from one cell. The technique can include isolation of tissues of a plant organ such as a leaf base. Induction of embryogenic cells and regeneration the cells will then be conducted. This was then followed by selection of plants which have the same characteristics as Si Madu in the field, especially after harvest. Another selection technique that can be done is detection of planting material, such as *in vitro* culture, using isozyme or RAPD markers. Finally, cultures or plants that have the same characteristics as their mother plant (Si Madu) are main-tained and propagated by either the conventional technique or by the *in vitro* technique such as organo-genesis.

CONCLUSION

Regeneration of pineapple *in vitro* cultures can be done by organogenesis and embryogenesis techniques. These techniques are applicable for propagation, conser-vation, and improvement of pine-apple. The embryogenesis tech-nique is potential for separating chi-mera cells and maintaining mutant cells of pineapple cultivar Si Madu from Subang, West Java. The non-chimera plants can either be propa-gated by using conventional or organogenesis technique.

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