RESPONSE OF FIVE KENAF ACCESSIONS TO SHOOT REGENERATION

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ABSTRACT

This experiment was aimed to study response of fives kenfa accession to shoot regeneration and to establish an appropriate regeneration protocol for kenaf. The experiment was performed at Tissue Culture Laboratory Research Institute for Tobacco and Fibre Crops (RITFC), Malang, from June to October 1997. Cotyledons with plumules attached were used as explants and culture medium for callus induction was MS-based medium with BAP (2 mg/l) and NAA (0.5 mg/l). Calli produced in this cultures were transferred into MS-based medium containing BAP (2 mg/l) and GA_3 (5 mg/l) for shoot initiation. All shoots obtained were then sub-cultured in MS-based medium without regulators (MS0) for root formation. Rooted shoots (plantlets) were acclimatized in the sterile sand and transferred into sterile soil in the glass house. Results of this experiment showed that the most responsive accessions were Cuba 108/I, followed by KK 60, Hc 48, PI 324922, and CHN/ 056 H with the average number of shoots per explant 4.32 ± 4.21, 4.00 ± 4.01, 3.05 ± 2.98, 2.80 ± 1.47, and 2.72 ± 2.49, respectively, at 40 days after transferring on shoot regeneration medium. Shoots rooted after 14 days on MS0 medium with frequencies of 81.50-93.30%. Healthy plantlets survived and grew well in soil media in the glass house. Therefore, an appropriate shoot regeneration protocol for kenaf was found.

Key words : *Hibiscus cannabinus* L., shoots regeneration, tissue culture

RINGKASAN

Respon lima aksesi kenaf terhadap regenerasi tunas

Penelitian ini bertujuan untuk mempelajari respon lima aksesi kenaf terhadap regenerasi tunas dan menemukan metode regenerasi yang sesuai untuk kenaf. Penelitian dilaksanakan di laboratorium Kultur Jaringan, Balai Penelitian Tembakau dan Tanaman Serat (Balittas), Malang, mulai Juni - Oktober 1997. Kotiledon beserta plumulenya digunakan sebagai eksplan. Media yang digunakan untuk induksi kalus adalah MS dasar dengan BAP (2 mg/l) dan NAA (0,5 mg/l). Kalus yang dihasilkan pada media ini kemudian dipindahkan ke media inisiasi tunas yaitu MS dasar yang mengandung BAP (2 mg/l) dan GA_3 (5 mg/l). Tunas yang diperoleh kemudian disub-kultur ke media MS dasar tanpa zat pengatur tumbuh (MS0) untuk pembentukan akar. Tunas yang telah berakar (planlets) kemudian diaklimatisasi pada media pasir steril dan ditanam pada tanah steril di rumah kaca. Hasil penelitian menunjukkan bahwa aksesi yang paling responsif adalah Cuba 108/I, diikuti oleh KK 60, Hc 48, PI 324922, dan CHN/ 056 H dengan jumlah tunas rata-rata per eksplan masing-masing: 4.32 ± 4.21, 4.00 ± 4.01, 3.05 ± 2.98, 2.80 ± 1.47, dan 2.72 ± 2.49; pada 40 hari setelah ditumbuhkan pada media induksi tunas. Tunas berakar pada 14 hari setelah dipindahkan ke media MS0 dengan persentase antara 81.50-93.30%. Planlet yang sehat tumbuh normal pada media tanah di rumah kaca. Dengan demikian metode regenerasi tunas yang sesuai untuk kenaf telah ditemukan.

Kata kunci : *Hibiscus cannabinus* L., tunas regenerasi, kultur jaringan

INTRODUCTION

Plant tissue culture has been applied widely to improve plant production in a broadest sense, for example in areas such as germplasm conservation, micro-propagation, advance breeding, and secondary metabolite production (HUSSEY, 1986; WITHERS, 1986; SCOWCROFT and RYAN, 1986; STAFFORD, 1991). Over the last decades, cell and tissue cultures have been commonly applied as the precious tools in genetic engineering or plant biotechnology, such as protoplast fusion to generate somatic hybrids or cybrids and plant transformation with specific genes. Genetic engineering in plant improvement is generally used as a supplement in conventional breeding, because this technique has a particular value in speeding up conventional breeding, reducing space and labour requirements or achieving manipulative goals that cannot be carried out *in vivo* (LINDSEY and JONES, 1990). A lot of successful works on plant transformation have been published, for instance potato transformation to obtain cultivar which is resistance to Potato Virus X (XU *et al.*, 1995) and herbicide resistance (FILHO *et al.*, 1994).

Meanwhile, reports of tissue culture on bast fibre crops, especially kenaf (*Hibiscus cannabinus*) are very limited. Some basic tissue culture works of bast fibre crops have been studied by scientists in Bangladesh, but mainly on jute (*Corchorus olitorius* and *C. capsularis*) (KHATUN *et al.*, 1996). Shoot regeneration of jute has been successfully achieved from leaf and shoot-tip explants (HAQUE *et al.*, 1992). KHATHUN *et al.* (1993) informed that the non-ionic surfactant (Pluronic F-68) supplemented in culture medium with 0.001-0.5% (w/v) stimulated shoot production of *C. capsularis* var. D 154 petioles and C 134 cotyledons. Therefore, it is necessary to establish an efficient tissue culture or shoot regeneration protocol of kenaf to allow application of genetic manipulation for varietal improvement. In this study, shoot regeneration of kenaf was performed through callus initiation, because callus is formed by continuous cell division at the cut surface of plant tissues which are explanted and supplied with a mixture of salts, vitamins, growth regulators, and carbon source. This technique produces more shoots than direct shoot regeneration from explant. The production of more shoots gives an advantage in varietal improvement, especially for the application of specific gene transfer technology. The objectives of this experiment were to study responses of five accessions of kenaf to shoot regeneration system, and to develop shoot regeneration protocol suitable for five accessions of kenaf.

MATERIALS AND METHODS

This experiment was conducted in Tissue Culture Laboratory, Research Institute for Tobacco and Fibre Crops

(RITFC), Malang, from June to October 1997. Five accessions of kenaf (*Hibiscus cannabinus*, L) were used in this experiment, namely Cuba 108/I, KK 60, Hc. 48, PI 324922, and CHN/056 H. Cotyledons with plumules attached of 7 days old seedlings were used as explants. The experiment was comprised of four steps as follows:

Seedlings

This step was carried out in June 1997. Kenaf seeds obtained from germplasm collection of Research Institute for Tobacco and Fiber Crops (RITFC) were surface disinfected by immersion in 90% (v/v) ethanol for 10 minutes and 30% (v/v) commercial hypochlorite solution for 30 minutes, followed by rinsing 5 changes of sterile distilled water. Seeds were germinated on the surface of 30 ml aliquots of agar-solidified (0.7% w/v) MS-based medium without growth regulators (MS0), in 175 ml capacity glass jars. Seeds were incubated in 25-28°C room under 16-h photoperiod for 7 days. The seedlings produced in this step were used as explant sources of the next step.

Callus Induction

This step was conducted in June and July 1997. Cotyledons of kenaf with their plumules excised from 7-day-old seedlings were used as explants. They were cultured in 50 ml capacity jars containing 20 ml aliquots of agar-solidified (0.7% w/v) MS-based medium. Plant growth regulators, namely BAP (2mg/l) and NAA (0.5 mg/l) were added to the medium for callus induction. Each accession consisted of 20 jars with a pair of cotyledons per jar. Cultures were incubated at 25-28°C with a 16-h daylength for 40 days. At 40 days after culture, all accessions produced shoots and calli. Number of shoots per explant obtained in this step were recorded and then sub-cultured to the MS-based medium without of growth regulators (MS0) for rooting. Meanwhile, the calli were used for shoots regeneration.

Shoots Regeneration

Shoots regeneration step was performed in July and August 1997. Calli produced in the previous step were subcultured to the shoot formation medium i.e. MS-based medium containing BAP (2 mg/l) and GA_3 (5 mg/l). Each accession consisted of 20 jars with a callus per jar. Cultures were incubated at 25-28°C with a 16-h daylength for 40 days. Number of shoots per callus regenerated on this medium were recorded at 40 days after sub-culturing. The shoots were then transferred to the MS0 medium for rooting.

Root Formation and Acclimatization

This step was performed from September to October 1997, the shoots achieved in step 2 and 3 were transferred to the MS0 medium for rooting and incubated at 25-28°C room with a 16-h daylength for 14 days. Acclimatization was done by transferring rooted shoots to sterile sand medium and incubating them at 25-28°C for 7 days, then transferred to the pot containing sterile soil, in the glasshouse.

RESULTS AND DISCUSSION

Callus Induction

Cotyledons with plumules attached of 5 accessions produced normal shoots as well as calli on callus induction medium: MS + 2 mg/l BAP + 0.5 mg/l NAA (Figure 1). Shoots were appeared within 7 days of culture. Meanwhile, calli formed within 14 days of culture at cut surface of cotyledons and basal of plumules. The percentage of explants produced shoots and calli, and the average number of shoots per explant of five accessions at 40 days of culture are presented in Table 1.

It was clear that all explants of all accessions produced calli easily (100%). The ease of calli production on five accessions was caused of the continuously cell division at the cut surface of the tissues, which were explanted on the medium suitable for callus induction (LINSEY and JONES, 1990). Shoots were regenerated directly from plumules, but regeneration was slightly difficult, especially on Cuba 108/I, KK 60 and CHN/056 H, since the medium was for callus initiation.

Table 1. Percentage of explant produced shoots and calli, and the average number of shoots per explant at 40 days after culturing
Tabel 1. Persentase eksplan yang menghasilkan tunas dan kalus, dan jumlah rata-rata tunas per eksplan 40 hari setelah kultur

Accessions *Aksesi*	% explant produced shoots *% eksplan menghasilkan tunas*	% explant produced calli *% eksplan menghasilkan kalus*	No. of shoots per explant*) *Jumlah tunas per eksplan*	CV of No. of shoots per explant *Jumlah variasi koefisient per eksplan*
				%
Cuba 108/I	94.70	100.00	1.72 ± 1.36	79.10
KK 60	70.00	100.00	1.64 ± 0.74	45.10
Hc 48	100.00	100.00	1.16 ± 0.37	31.90
PI 324922	100.00	100.00	1.32 ± 0.48	36.40
CHN/056H	93.30	100.00	0.93 ± 0.26	28.00

Note : *) Average standard of deviation *Rata-rata standar deviasi*
Keterangan : CV: Coefficient of Variation *Variasi koefisien*

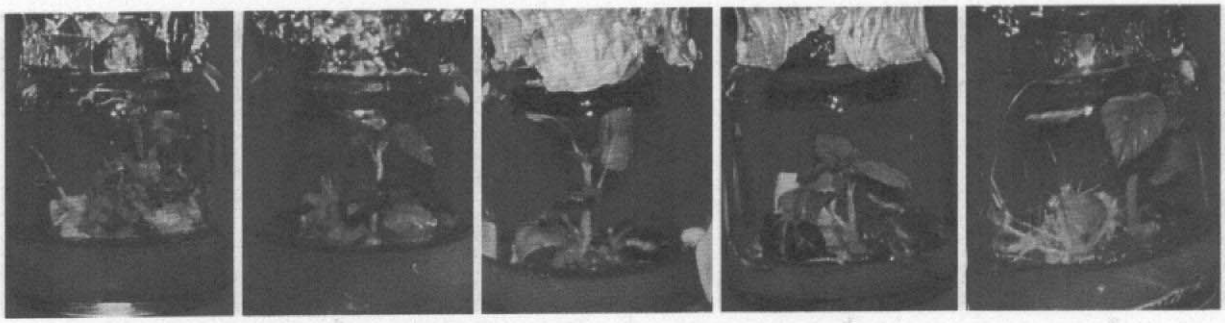

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Figure. 1 a-e. Cotyledons+plumules of kenaf accessions Cuba 108/I, KK 60, Hc 48, CHN/056 H, and PI 324922 at 40 days after culturing on MS-based medium + BAP (2 mg/l) + NAA (0.5 mg/l)

Gambar 1 a-e. Cotyledon + plumule aksesi kenaf Cuba 108/1, KK 60, Hc 48 CHN/056 H, dan PI 324922 pada 40 hari setelah kultur dalam MS media + BAP 92 mg/l) + NAA (0.5 mg/l)

On this medium, explants of PI 324922 produced a great number of roots (Figure. 1e), meanwhile the other four accessions (Cuba 108/I, KK 60, Hc 48, and CHN/056H) did not. This condition could be caused of the endogenous auxin of PI 324922 was already enough for callus formation, so that in addition of auxin (NAA) to the medium would stimulate roots induction. Similar result was reported by HANDAJANI (1996) i.e. hipocotyl of kenaf could produce callus on MS-based medium without auxin. The addition of 2 – 6 mg/l auxin (IAA and IBA) on culture medium formed a lot of roots. ALLAN (1991) demonstrated that it was simple relationship between the auxin-cytokinin balance of the nutrient medium, and the pattern of redifferentiation of unorganized callus. If the cytokinin concentration was high relative to that of auxin, then shoot development was induced; whereas if the auxin concentration was relatively high, then root would develop. At intermediate concentrations, the tissue developed as an unorganized callus. Moreover, NICKELL (1982) reported that NAA is one of the commonly used chemical for rooting initiation, because this compound is decomposed relatively slowly by the auxin-destroying enzyme systems and moves very slowly in the plant tissues, much of it is retained near the site of application.

Shoot Regeneration

At 40 days after sub-culturing on MS-based medium containing BAP and GA_3 (shoot regeneration medium), calli of all accessions produced shoots with average number per explant as follows : Cuba 108/I (4.32 ± 4.21), KK 60 (4.00 ± 4.01), Hc 48 (3.05 ± 2.98), PI 324922 (2.72 ± 2.49), and CHN/ 056 H (2.80 ± 1.47) (Table 2 and Figure 2). Four out of five accessions tested show high values of standard of deviation with the CV 68% (Cuba 108/1: 97.50%, KK 60 : 100.25%, Hc 48 : 97.70%, and PI 324922 : 91.50%). This means that the ability to regenerate shoots of each explant on each accession was varied, (KOOPMANS, 1987) elucidated when the value of CV is > 68%, the data are not in a normal distribution. Cuba 108/I is the most responsive accession to produce shoots followed by KK 60, Hc 48, PI 324922 and CHN/056 H. The variation of shoot production on each accessions and response of each accession to shoot regeneration protocol employed in

Table 2. Percentage of calli produced shoots and number of shoots per explant of 5 accessions of kenaf on shoot regeneration medium

Tabel 2. *Persentase kalus yang menghasilkan tunas dan jumlah tunas per eksplan pada medium perbanyakan dari 5 aksesi kenaf*

Accessions *Aksesi*	% of calli produced *% kalus menghasilkan tunas*	No. of shoots per explant *) shoots *Jumlah tunas per eksplan*	CV of No. of shoots per explant *Jumlah variasi koefisien tunas per eksplan*	Total of shoots obtained**) *Total tunas yang dihasilkan*
				%
Cuba 108/I	98.00	4.32 ± 4.21	97.50	6.04
KK 60	85.00	4.00 ± 4.01	100.25	5.64
Hc 48	100.00	3.05 ± 2.98	97.70	4.21
PI 324922	100.00	2.72 ± 2.49	91.50	4.04
CHN/056H	96.00	2.80 ± 1.47	52.50	3.73

Note : *) Average standard of deviation

Keterangan : Rata-rata standar devisiasi

**) Number of shoots which were produced on callus induction medium + shoot regeneration medium

Jumlah tuna yang dihasilkan dalam medium induksi kalus dan medium

Figure. 2 a-e. Derived shoots from calli of kenaf accessions Cuba 108/I, KK 60, Hc 48, CHN/056 H, and PI 324922 at 40 days after transferring on MS-based medium + BAP (2 mg/l) + GA_3 (5 mg/l)

Gambar 2 a-e. Tunas yang berasal dari kalus aksesi kenaf Cuba 108/1,KK 60, Hc 48, CHN/056 H, dan PI 324922 pada 40 hari setelah dipindahkan ke dalam medium MS + Bap (2 mg/l0 + GA_3 (5 mg/l)

this experiment was probably affected by their genotype. HULME *et al.* (1992) similarly found that there was an interaction between genotype and the specific regeneration technique used. PHILLIPS and COLLINS (1979) reported that four cultivars (genotypes) of red clover produced different responses in plant regeneration treatments. DAVIDDONIS and HAMILTON (1989) observed that there was genotype dependent on plant regeneration from callus of cotton; they could regenerate cotton variety Coker 310 only. ABDULLAH *et al.* (1994) reported that callus induction and plant regeneration of diploid *Lotus* varied with genotype and explant type. PURWATI (1996) described a genotype effect on shoot regeneration of four cultivars of potato.

All accessions resulted in more shoots on MS-based medium containing BAP (2 mg/l)+GA_3 (5 mg/l) than BAP (2 mg/l)+NAA (0.5 mg/l). These results similar to KARP *et al.* (1984) who found that GA_3 is an effective plant growth regulator to stimulate shoot regeneration. They have successfully used GA_3 to regenerate potato shoots from leaf discs.

Root Formation and Acclimatization

Roots formation of five accessions tested was not too hard, it took 14 days after transfer to root induction medium (MS0). The frequency of rooted shoots was ranged from 81.50% to 93.30% (Table 3 and Figure 3). The same condition was found for acclimatization of rooted shoots (plantlets) to the sterile sand and soil (Table 3).

Table 3. The percentage of shoots rooted on MS0, and plantlets survived on sand and soil media

Tabel 3. Persentase tunas yang berakar dalam MS0 dan Plantlet yang hidup dalam media pasir dan tanah

Accessions *Aksesi*	% shoots rooted *5 tunas berakar*	% plantlets survived on *% plantlet yang hidup dalam pasir steril*	% plantlets survived on sterile soil sterile sand *% plantlet yang hidup dalam tanah steril*
Cuba 108/I	81.50	82.70	85.70
KK 60	93.30	94.70	95.30
Hc 48	83.30	84.30	90.70
PI 324922	83.30	85.30	90.30
CHN/056H	85.00	86.70	92.30

Healthy plantlets grew well in the glasshouse (Figure 4). There was no visual differences plants derived from calli and seeds.

CONCLUSION

In this experiment, the explants of cotyledons+plumules can regenerate shoots with average number 0.93-1.72 per explant on MS-based medium containing BAP (2 mg/l) and NAA (0.5 mg/l); and 2.80-4.32 on MS-based medium with BAP (2 mg/l) and GA_3 (5 mg/l). Total shoots obtained on both media ranges 3.73-6.04 per explant. Plantlets were obtained within 54-94 days. Shoots rooted easily on medium MS0, and acclimatization of the plantlets was not too difficult, since 85.70-95.30 % grew well in the glasshouse.

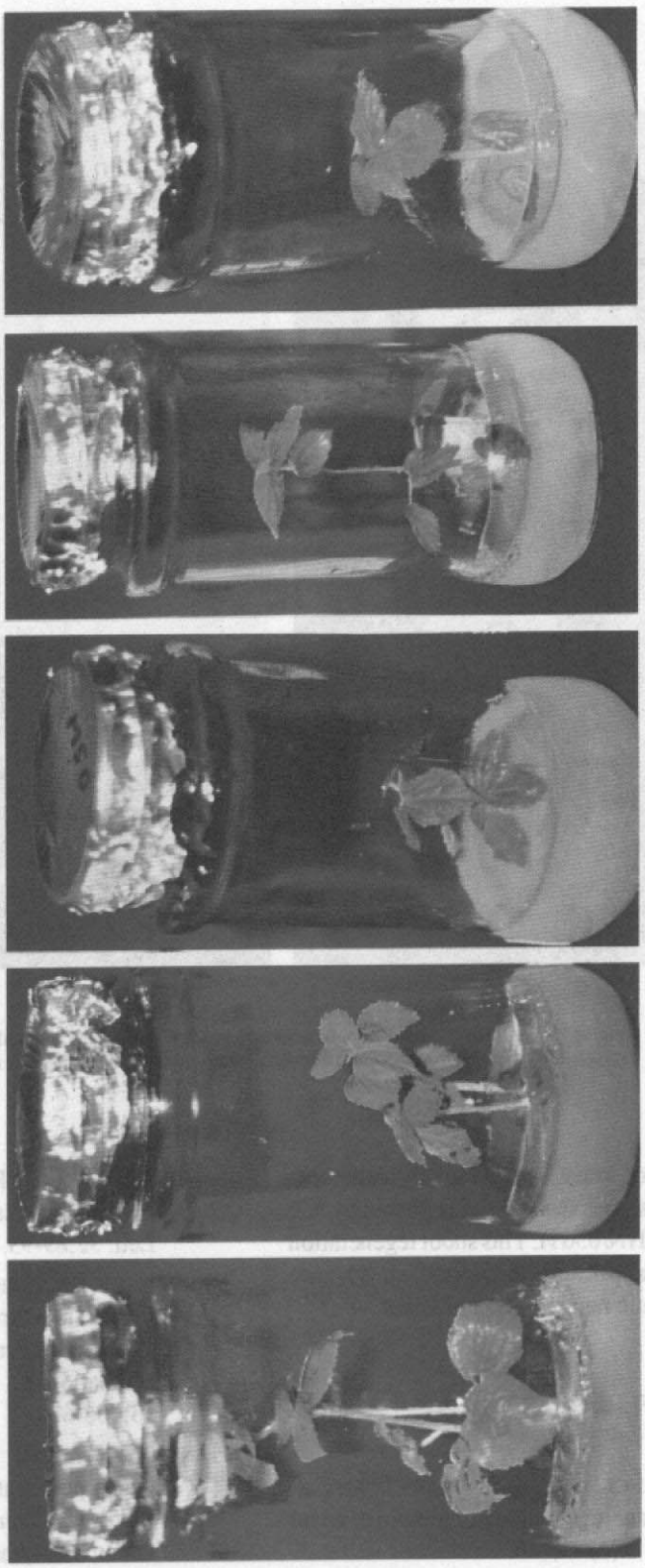

Figure 3 a-e. Shoots of accesions Cuba 108/1, KK 60, Hc 48, CHN/056 H, and PI 324922 rooted on MS-based medium lacking growth regulators (MS0)

Gambar 3 a-e. Tunas aksesi kenaf Cuba 108/1, KK 60, Hc 48, CHN/056 H, dan PI 324922 yang berakar dalam medium MS tanpa zat pengatur tumbuh (MS0)

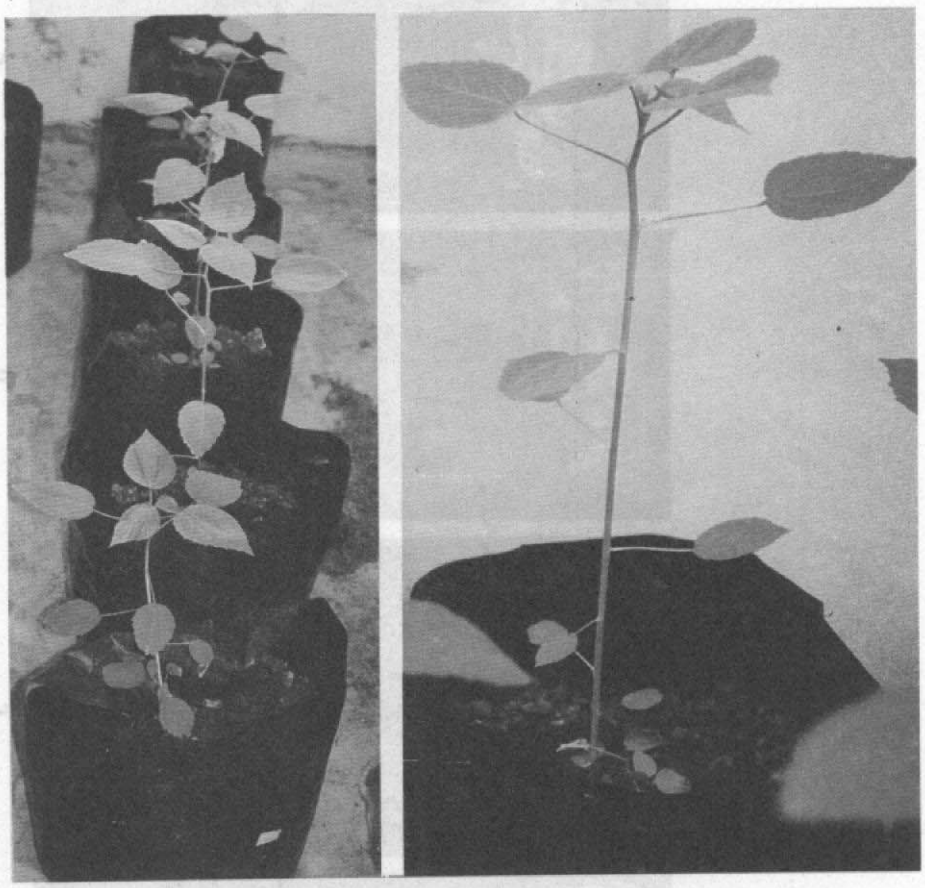

Figure 4. Plants obtained from *in vitro* culture grow well on the soil in the glass house
Gambar 4. Tanaman yang dihasilkan dari kultur in vitro tumbuh dengan baik dalam media tanah di rumah kaca

Shoots regeneration of kenaf is affected by genotype. The most responsive accession is Cuba 108/I followed by KK 60, Hc 48, PI 324922 and CHN/056 H. This shoot regeneration protocol is suitable and applicable to support an advance breeding program by genetic manipulation of kenaf.

REFERENCES

ABDULLAH, B., P.R. BEUSELINCK, and R.L. MCGRAW. 1994. Callus induction and plant regeneration of diploid of *Lotus* species. Zuriat Vol. 5 (2): 38-44.

ALLAN, E. 1991. Plant Cell Culture. In : Stafford, A and Warren, G (eds.) Plant Cell and Tissue Culture. Open University Press, Milton Keynes, England, pp 1-24.

DAVIDDONIS, G.H. and R.H. Hamilton. 1989. Plant regeneration from callus tissue of *Gossypium hirsutum*. Plant Sci. Lett. 32:89-93.

FILHO, E.S.F. L.F.A. FIGUEIREDO, and D.C. MONTE-NESHICH. 1994. Transformation of potato (*Solanum tuberosum*) cv. Mantiqueira using *Agrobacterium tumefaciens* and evaluation of herbicide resistance. Plant Cell Rep. 13:666-670.

HANDAJANI, F. 1996. Pengaruh berbagai macam auksin terhadap pembentukan kalus pada kultur jaringan kenaf (*Hibiscus cannabinus*, L). Skripsi untuk Sarjana Pertanian. Fakultas Pertanian, Universitas Wijaya Kusuma, Surabaya. 81 p.

HAQUE, M.M., HAQUE, I., and SARKAR, R.H. 1992. Tissue culture as a tool in jute improvement-status, impact and future

potential. Proceeding of the IJO/BJRI Training Course on Specialized Techniques in jute and kenaf breeding. BJRI, Dhaka, Bangladesh. pp.287-304.

HULME, J.S. E.S. HIGGINS and R. SHIELD. 1992. An efficient genotype-independent method for regeneration of potato plants from leaf tissue. Plant Cell, Tissue and Organ Culture, 31,161-167.

HUSSEY, G. 1986. Vegetative propagation of plants by tissue culture. *In:* Yeoman, M.M. (ed.) Botanical Monographs Vol. 23. Plant Cell Culture Technology. Blackwell Scientific Publications, Oxford, pp. 27-66.

KARP, A., R. RISSIOTT, M.G.K. JONES, and S.W.J. BRIGHT. 1984. Chromosome doubling in monohaploid and dihaploid potatoes by regeneration from cultured leaf explants. Plant Cell, Tissue and Organ Culture 3, 363-373.

KHATUN, A., L. LAOUAR, M.R. DAVEY, J.B. POWER, B.J. MULLIGAN, and K.C. LOWE. 1993. Effects of Pluronic F68 on shoot regeneration from cultured jute cotyledons and on growth of transformed roots. Plant Cell, Tissue and Organ Culture 34,133-140.

KHATUN, A. 1996. Recent advances in biotechnological research in jute. Paper presented in Regional Training Course on Jute and Kenaf breeding of the IJO "Support for varietal improvement of jute and kenaf". BJRI, Dhaka, 5-13 August 1996. 14p.

KOOPMANS, L.H. 1987. Introduction to contemporary statistical methods. Duxbury Press. Boston, p. 683.

LINDSEY, K., and M.G.K. JONES. 1990. Plant Biotechnology in agriculture. Prentice Hall, Englewood Cllifs, New Jersey, pp 1-241.

NICKELL, L.G. 1982. Plant growth and regulators, Agricultural uses. Springler-Verlag, Berlin, p. 173.

PHILLIPS, G.C. and G.B. COLLINS. 1979. In vitro tissue culture of selected legumes and plant regeneration from callus cultures of red clover. Crop. Sci. 19:59-64.

PURWATI, R. D. 1996. Potato transformation using *Agrobacterium tumefaciens* to study root-knot nematode (*Meloidogyne* spp.) resistance. Master of Philosophy Thesis. State Agricultural and Biotechnology Centre, School of Biology and Environmental Sciences, Murdoch University, Perth, Western Australia.97p.

SCOWCROFT, W.R. and S.A. RYAN. 1986. Tissue culture and plant breeding. *In:* Yeoman, M.M. (ed.) Botanical Monographs Vol. 23. Plant Cell Culture Technology. Blackwell Scientific Publications, Oxford, pp. 67-95.

SNYDER, G.W. and W.R. BELKNAP. 1993. A modified method for routine *Agrobacterium*-mediated transformation of *in-vitro* grown potato microtubers. Plant Cell Rep. 12: 324-327.

STAFFORD, A. 1991. Natural products and metabolites from plants and plant tissue cultures. *In* : Stafford, A and Warren, G (eds.) Plant Cell and Tissue Culture. Open University Press, Milton Keynes. England, pp 124-162.

WITHERS, L.A. 1986. Cryopreservation and gene banks. *In*: Yeoman, M.M. (ed.) Botanical Monographs Vol. 23. Plant Cell Culture Technology. Blackwell Scientific Publications, Oxford, pp. 96-142.

XU, H., H. KHALILIAN, M. EWEIDA, S. SQUIRE, and M.A. ABOUHAIDAR. 1995. Genetically engineered resistance to Potato Virus X in four commercial potato cultivars. Plant Cell Rep. 15:91-96.