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Direct somatic embryogenesis and plant regeneration in tea by temporary liquid immersion

Embriogenesis somatik langsung dan regenerasi tanaman teh melalui perendaman sesaat

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Ringkasan

Perbanyakan tanaman teh [Camellia sinensis (L.) O. Kuntze] *melalui stek tunas berdaun tunggal hanya dapat menghasilkan klon unggul dalam jumlah terbatas. Oleh sebab itu diperlukan metode alternatif dengan teknik kultur sel dan jaringan untuk perbanyakan klonal secara cepat. Dalam penelitian ini dikembangkan metode yang lebih efektif untuk regenerasi tanaman teh melalui embriogenesis somatik langsung. Massa proembriogenik dari eksplan kotiledon dihasilkan dengan frekuensi 56,7% dalam media MS padat setengah konsentrasi yang mengandung* BAP 2 *mg1L. Proliferasi, perkembangan, pendewasaan dan perkecambahan embrio somatik diperoleh dengan sistem perendaman sesaat (SPS) yang menggunakan media MS cair setengah konsentrasi, yang diperkaya dengan zat pengatur tumbuh dengan berbagai konsentrasi. Proliferasi embrio meningkat 4,3 kali dalam media yang diberi* BAP *2 mglL; perkembangan dan pendewasaannya meningkat dengan penambahan kinetin dan* ABA *masing-masing pada konsentrasi 0,1 mg1L yang 30% diantaranya berkecambah dan membentuk planlet tanpa penambahan zat pengatur tumbuh. Protokol SPS tersebut merupakan sistem* in vitro *yang berpotensi bagi proliferasi dan perkembangan embrio somatik tanaman teh yang cepat dan sinkron dari kultur kotiledon, serta regenerasinya menjadi planlet tanpa melalui fase kalus.*

Summary

Tea propagation by single-leaf bud cuttings has limited applications for rapid dissemination of planting materials from new elite clones. An alternative method for rapid cloning by cell and tissue culture technique is necessary. In this study we have established an improved method for tea *[Camellia sinensis (L.)* O. Kuntze] plant regeneration via direct somatic embryogenesis. Clumps of proembryogenic masses were initiated at a frequency of 56.7% from cotyledonary slices cultured on a half-strength MS agar-gelled medium supplemented with 2 mg/L BAP. Proliferation, development, maturation and germination of somatic embryos were achieved using the temporary immersion system (TIS) provided with half-strength MS liquid media supplemented with varying concentrations of growth regulators. Embryo proliferation increased by 4.3-fold in medium provided with 2 mg/L BAP; their development and maturation were enhanced by the presence of both kinetin and ABA at 0.1 mg/L each.

Germination and plant recovery were achieved at a frequency of about 30% without the use of growth regulators. The TIS protocol described above represents an *in vitro* system potential for rapid proliferation and synchronized development of tea somatic embryos from cotyledon cultures, and their regeneration into plantlets without an intervening callus phase.

[*Keywords:* Somatic embryogenesis, plant regeneration, synchronization, temporary immersion, tea, *Camellia sinensis (L.)*]

Introduction

Cultivated tea *[Camellia sinensis (L.) O.* Kuntze] is conventionally propagated by single leaf-node cuttings. While planting materials produced by such method are relatively inexpensive, the process in establishing tea plants from cuttings is lengthy, labour-intensive and not amenable to rapid mass multiplication. Propagation through proliferation of axillary buds *in vitro* offers a faster method of clonal multiplication (Nakamura, 1991; Agarwal *et al.,* 1992; Tahardi, 1994) even though it may not be efficient for large-scale commercial application. Recently, research on tea micropropagation has been focused on exploring the potential of somatic embryogenesis as a more efficient means of plant multiplication and regeneration. Bano *et* al. (1991), Wachira & Ogada (1995) and Ponsamuel *et* al. (1996) reported induction of somatic embryogenesis in tea, from cotyledonary explants cultured on agar-gelled media. However, in all these studies, the development, maturation and germination of somatic embryos were not well synchronized, resulting in low plant uniformity.

Synchronization of embryo development is essential for efficient production of uniform propagules *in vitro.* Recently, a novel *in vitro* culture system based on short periodic immersion of explants in a liquid medium has been tested successfully for the synchronized growth of embryogenic cultures of coffee, *Hevea* rubber (Teisson & Alvard, 1995), *citrus* (Cabasson *et al.,* 1997) and oil palm (Tahardi, 1998).

In this paper, we report the application of the temporary immersion system (TIS) for inducing direct somatic embryogenesis and plant regeneration from cotyledonary cultures of tea of Yabukita variety. It is also hoped that through this model culture system, a better understanding of the regulation of somatic embryo development, germination and plant conversion can be obtained.

Materials and Methods

Tea seeds from moderately mature fruits (Figure la) used in this investigation were collected from mother shrubs of *Camellia sinensis (L),* variety Yabukita grown at the Pasir Sarongge Tea Experimental Station, Cipanas, West Jawa. The greenish-brown fruits were washed in running tap water and rinsed briefly in 70% ethanol. After removal of the fruit pods, the surface of the seeds was disinfected in 20% (v/v) commercial bleach for 15 min, and then rinsed several times with sterile distilled water. The embryos were excised from the seeds, and the cotyledons cut into 5-mm slices and placed into Petri plates containing a half-strength Murashige-Skoog (MS) basal medium containing 30 g/L sucrose and supplemented with benzylaminopurine (BAP) at 0, l , 2, 5 and 10 mg/L The medium was solidified with

0.2% Gelrite after pH adjustment to 5.8 and autoclaved at 121°C and 105 kPa for 20 min. The cultures were kept in the dark at 25 °C until initiation of proembryos.

Proembryos of uniform size and stage were selected for further proliferation and development in the same half-strength MS basal medium supplemented with 2 mg/L BAR In order to evaluate the efficiency of embryo proliferation and development, three different culture systems, namely the semisolid, the shake-flask and the temporary immersion system (TIS) were used. Clumps of proembryogenic mass with developing globular embryos weighing about 1 g were inoculated into the respective cultures and their development followed through two successive culture periods of 4 weeks each.

Further studies on the development, maturation and germination of tea somatic embryos were subsequently carried out in the TIS. The TIS apparatus is basically a commercial liquid filter unit (Nalgene, Nalge Co., USA), modified by connecting the upper and the lower compartment by means of a small glass tube fitted to a screen disc at the bottom of the upper compartment (Figure lc). Clusters of proembryos of relatively uniform size weighing about 2 g were placed on a screen disc in the upper compartment, and about 125 mL of a half-strength MS liquid medium containing 20 g/L sucrose and hormonal supplements such as kinetin, gibberellic acid (GA,) and abscisic acid (ABA) at 0.1 mg/L each were placed in the lower compartment. When the lower compartment was pressurised, the nutrient solution was pumped from the bottom into the upper compartment. For embryo development, immersion of the proembryogenic mass was programmed for 3 min each, at intervals of 6 h, and for embryo maturation, at intervals of 24 h. The cultures in triplicates were incubated at 27 °C under a 12-h photoperiod with diffuse lighting providing an intensity of 30 gmoles photons/ M'/sec.

The cultures were monitored weekly using a magnifying glass for proliferation, development, maturation and germination of somatic embryos. Embryo counts based on developmental stages were conducted aseptically every 3-4 weeks at the time of subculturing using a Zeiss binocular microscope set at 8X magnification.

Cotyledonary-stage somatic embryos of uniform size were germinated under normal TIS conditions (daily immersion of 3 min each, at intervals of 6 h) in a half-strength MS liquid medium without growth regulators. Regenerated plantlets were transferred to a half-strength MS medium solidified with 0.2% Gelrite and incubated with regular subculturing every 4-6 weeks until plantlets with stout leafy shoots and tap roots were obtained.

Results and Discussion

Induction of somatic embryos from tea cotyledon explants

One week after culture initiation, the deembryonated cotyledonary slices began to swell, forming compact, bulging tissue masses of varying shape and size. Table 1 shows the effects of BAP on the induction of embryogenic cultures. The frequency of embryo formation ranged from 0 to 56.7% after 4 weeks of culture in half-strength MS media supplemented with BAP from 0 to 10 mg/L. Somatic embryos were formed directly from the cotyledon surface, with very little or no callusing at all. At higher concentrations of BAP (5 and 10 mg/L), callusing was more conspicuous but no somatic embryos were initiated from the, apparently non-embryogenic friable calli. In cultures which were embryogenic, the bulging tissue masses later gave rise to numerous globular embryos (Figure lb).

Table 1. Effects of BAP on the induction of embryogenic cultures from cotyledon explants of tea.

BAP (mg/L)	No. cultured	No. explants embryogenic	Embryogenic (%)
0	30	10	33.3
1	30	12	40.0
2	30	17	56.7
5	30	0	0
10	30	0	0

The induction of somatic embryos from cotyledonary cultures appears to be regulated by growth hormones such as cytokinin and auxin, alone or in combination. Kato (1996) reported the predominant use of cytokinin (BA) in establishing embryogenic cotyledonary cultures from two tea species without an intermediate callus phase. On the other hand, Bano *et al. (1991)* and Wachira & Ogada, (1995) using auxin (2,4-D) predominantly, also succeeded in obtaining somatic embryos from cotyledons via a callus phase. In other instances, somatic embryos were obtained from immature cotyledons without supplementation of growth regulators. A plausible explanation for this phenomenon might be the presence of endogenous hormones at a level sufficiently effective for induction of somatic embryos in young cotyledonary tissues.

The levels of response of cotyledonary tissues to embryogenic induction by exogenous hormones have been shown to be correlated to the stage of seed maturity. Bano *et al.* (1991) reported that tender (immature) cotyledons yielded the highest frequency of embryogenic cultures in MS medium with 0.5 mg/L 2,4-D and 0.05 mg/L kinetin. In the present experiment however, embryogenic cultures were established from moderately mature cotyledonary explants in half-strength MS basal media supplemented with 1 and 2 mg/L BAP alone or without growth regulator. This result is in line with the observations by Nakamura (1988) that cytokinin alone promoted induction of direct somatic embryogenesis from cotyledonary explants in tea. Such cytokinin-induced embryogenic cultures generally tend to give rise to fewer morphological types in contrast to embryogenic cultures induced by a high auxin to cytokinin concentration.

Proliferation ofsomatic embryos

In order to improve the efficiency and synchrony of embryo proliferation, the pro-embryogenic masses from the cotyledonary tissues were further cultured in the same medium using three different culture systems (semi-solid, shake-flask and TIS). During the first 4 weeks, all the three culture systems proved to be doing relatively well with respect to biomass increase and embryo proliferation (Table 2). However, during the second 4 weeks, or 8 weeks after culture initiation, the shake-flask system started to lag behind, with cultures showing substantially reduced gain in biomass and embryo proliferation. In the other two culture systems (semi-solid and TIS) however, embryo proliferation and growth were still maintained at high rates. While the performance of the two culture systems - was not significantly different from each other, the temporary immersion system appeared to outperform the semi-solid

Table 2. Effects of culture systems on the growth of proembryogenic masses and proliferation of somatic embryos derived from cotyledonary cultures

Culture system[1]	Fresh weight(g)			No. developing embryoso		
	0	4	8 (wk)	0	4	8 (wk)
Semi-solid	1	2.597	5.947	102	192	402
Shake-flask	1	2.489	3.456	96	120	198
TIS	1	2.161	5.781	84	138	360

I Media used for the three culture systems were half-strength MS supplemented with 2 mg/L BAR Figures are averages of 10 replicates for the semi-solid, five for the shake-flask and three for the TIS culture.

culture system, particularly with respect to the synchronization of embryo proliferation and growth.

Somatic embryos regenerated under the shake-flask and the TIS conditions during the two culture periods (8 weeks) were mostly globular, whereas those produced on the semi-solid medium were of varying developmental stages. The emergence of predominantly globular embryos in the shake-flask and the TIS cultures reflects a synchronized morphogenic response as a result of uniform exposure of the proembryogenic tissues to the liquid nutrient medium (Teisson & Alvard, 1995). While the shake-flask system offers a homogenous culture environment, the lack of dissolved oxygen in the medium could pose a serious limitation to rapid growth, which could account for' the relatively low rate of embryo proliferation observed. Furthermore, the proliferating embryos were larger and tended to stay in clumps. In contrast, embryo proliferation and development on the semi-solid agar medium were asynchronous because growth of the proembryogenic masses had led to a rapid development of nutritional and hormonal gradients, resulting in an uneven exposure of the cultures to hormonal treatments (Osuga & Komamine, 1994).

Development and maturation of somatic embryos

Development and maturation of globular proembryos in the temporary liquid immersion system appeared to be influenced by a combination of growth regulators such as kinetin, gibberellic acid and abscisic acid. After 4 weeks of culture the mean increase in the biomass of developing embryos ranged from 3.937 to 6.285 g (2.76- to 3.85-fold) in media supplemented with two or more of these hormones, leading to a corresponding increase in the number of somatic embryos (Table 3). In the hormone-free medium, the mean increase in biomass was lower (3.396 g or 2.54-fold), with relatively fewer somatic embryos produced even after two successive cultures. This indicates that embryo proliferation still persisted in the development and maturation media during the first 4 weeks, particularly in media where kinetin and ABA or together with GA, were present. However, when the cultures were subsequently subjected to a

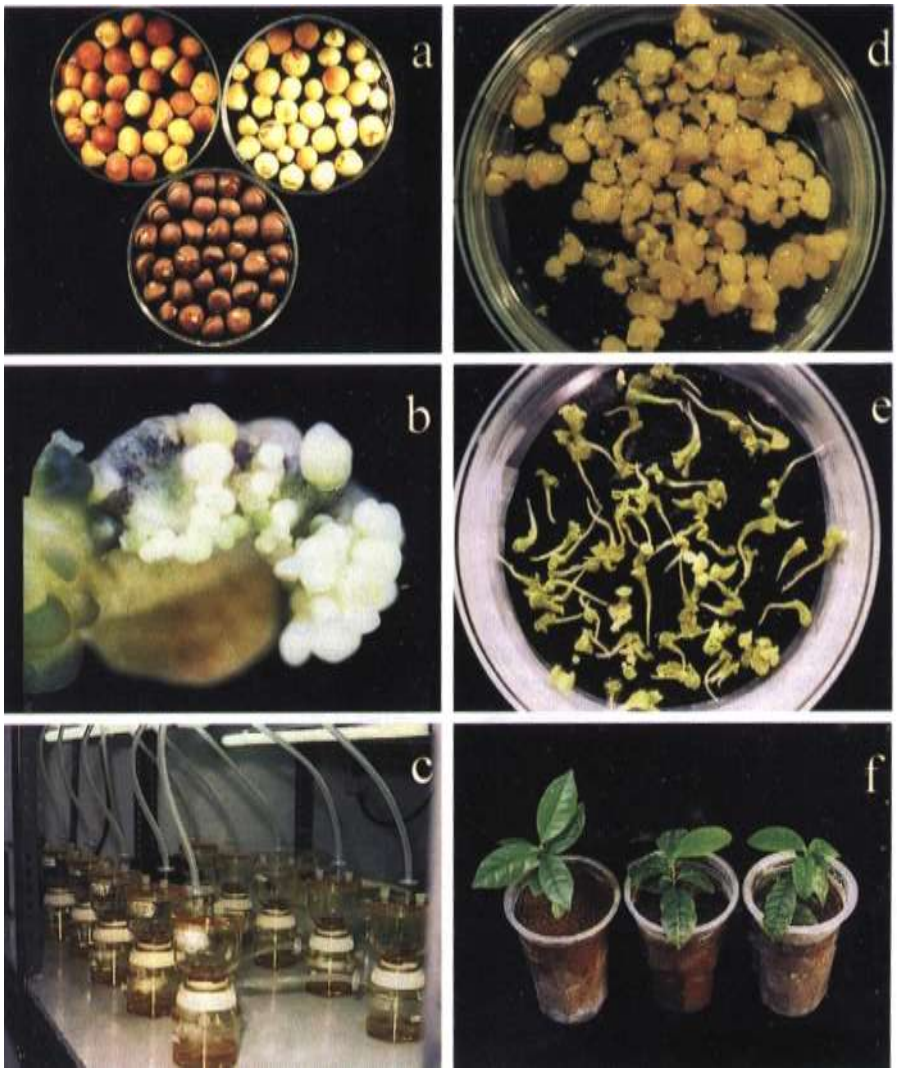

Figure l a-f Somatic embryogenesis and plant regeneration *in Camellia sinensis (L.)*, variety Yabukita (a) Moderately mature tea seeds, upper left; (b) Primary somatic embryos developing on cotyledon tissue; (c) Temporary irmnersion system; (d) Synchronized development of somatic embryos; (e) Germinated embryos with distinct shoot and root poles; (f) Tea plants from cotyledon-derived somatic embryos after establishment in soil.

Table *3.* Effects of hormonal combinations on the development and maturation of cotyledon derived embryos under temporary immersion culture (TIS) conditions.

Hormonal combination			Fresh Weight.(g)			No. developing embryos		
Kin	GA (mg/L)	ABA	0	4 (wk)	7	0	4 (wk)	7
0	0	0	2.203	5.599	5.929	217	235	236
0.1	0.1	0	2.235	6.172	6.215	250	277	281
0.1	0	0.1	2.207	8.492	11.15	225	402	421
0.1	0.1	0.1	2.241	7.280	9.151	270	326	335

Figures are averages of three replicates of TIS cultures

lower frequency of immersion (once every 24 h) during the second culture period, the increase in biomass and the number of developing embryos were relatively minor, suggesting that the embryo might have embarked into the maturation phase. Reducing the frequency of immersion had apparently limited the availability of nutrients and consequently induced some stress in the TIS cultures, triggering embryo maturation but reducing embryo proliferation.

Apart from the emergence of a few budding globular embryos, development and maturation of the primary globular embryos appeared to have been. reasonably well synchronized (Figure ld). More than 60% of the primary somatic embryos managed to progress into the later developmental stages after the seventh week (data not shown). A significantly higher developmental synchrony was attained in the presence of both kinetin and ABA, where more than 71.3% of the somatic embryos had progressed beyond the globular stage of development. The promotive effect of ABA at non-inhibitory levels on somatic embryo development and maturation has also been demonstrated in carrot (Kamada & Harada, 1981) and white spruce (Attree *et al.,* 1991). In this regard the role of ABA can also be ascribed to its ability to suppress secondary embryogenesis, thus allowing a synchronized development and maturation of the somatic embryos (Merkle *et al.,* 1995).

Germination and plantlet recovery

Germination of mature (cotyledonstage) somatic embryos was achieved at a rate of approximately 30% 3-4 weeks after transfer to a half-strength MS liquid basal medium under normal TIS culture conditions. Germinated embryos showed distinct shoot and root poles (Figure, l e). In other studies however, in the presence of plant growth regulators such as BA, IAA and GA, singly or in combination, the percentage of germination and plantlet conversion was significantly improved. Vieitez *et al.* (1992) reported a conversion rate as high as 40% in medium supplemented with 5 mg/L GA, and 2 mg/L IAA. Similarly, Wachira & Ogada (1995) obtained a germination percentage of about 60% in MS medium supplemented with 1 mg/L BAP or 1.8 mg/L kinetin. The stimulation of germination of tea somatic embryos and their conversion into plantlets as reported indicates a strong dependence on plant growth regulators for organ differentiation.

Plantlets regenerated from somatic embryos were successfully acclimatized and established in a pre-sterilized soil-sand (3:1) mix in the screenhouse with a low mortality rate. The tea plants derived from somatic embryos showed a true tap root, and resembled seedling-derived plants in form, growth rate and bud phenology (Figure 1 f).

Conclusions

The present study describes an effective protocol for rapid proliferation and development of somatic embryos from cotyledonary cultures of tea and their regeneration into plantlets using the temporary liquid immersion system. Embryo proliferation was achieved in a half-strength MS liquid medium provided with 2 mg/L BAP, followed by their development and maturation in the presence of both kinetin and ABA at 0.1 mg/L each. Germination and plant recovery were achieved in the same culture system without the use of growth regulators.

Compared to the agar-solidified and the shake-flask culture systems, the use of the temporary liquid immersion system in this study represents a unique contribution to improving the efficiency of the protocol, particularly with respect to embryo proliferation and their synchronized development. With appropriate scaling-up, this culture system may offer potential applications for mass production of clonal tea plants with tap roots via somatic embryogenesis.

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