

Morphological variations during the development of somatic embryos of tea (*Camellia sinensis* L.) *in vitro*

Keragaman morfologi selama perkembangan embrio somatik teh (Camellia sinensis L.) in vitro

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Ringkasan

Biak embrio somatik tanaman teh (*Camellia sinensis* L.) pada medium padat terdiri dari embrio dalam berbagai ukuran, warna dan stadia perkembangan. Satu gram embrio somatik yang sebagian besar dalam stadia globuler telah dibiakkan pada medium padat proliferasi (medium WP dengan IAA 57,1 μM dan BAP 4,4 μM) untuk mengamati keragaman morfologi embrio dalam hal ukuran, warna dan stadia perkembangan dalam satu periode kultur 6 minggu. Berat basah embrio somatik meningkat perlahan pada 4 minggu pertama kemudian meningkat dengan tajam. Pada minggu keempat, jumlah embrio melonjak walaupun beratnya tidak meningkat, hal ini menunjukkan adanya pembentukan embrio sekunder. Ukuran rata-rata embrio somatik tidak berubah secara nyata selama periode kultur, tetapi ukuran embrio sudah sangat beragam sejak awal kultur dan terus meningkat sejalan dengan berkembangnya embrio. Sekitar setengah dari embrio berwarna kuning dan sisanya terdiri dari embrio berwarna hijau dan merah. Pada awal kultur, 60% embrio berada pada stadia globuler, 30% stadia bentuk-hati dan 10% stadia bentuk-torpedo. Pada umumnya embrio globuler berkembang ke stadia lebih lanjut sejalan dengan waktu, tetapi pada medium proliferasi ini hampir 80% embrio masih dalam stadia globuler dan bentuk-hati pada minggu keenam. Apabila embrio somatik globuler tunggal dengan warna tertentu dibiakkan pada medium padat regenerasi (WP dengan kinetin 0,47 μM , ABA 0,69 μM dan GA_3 0,29 μM , sebagian embrio terutama embrio kuning akan mengalami perubahan warna. Sebagian besar embrio globuler tunggal ini

berkembang secara bertahap ke stadia perkembangan lebih lanjut. Warna awal embrio berpengaruh terhadap kecepatan perubahan stadia perkembangan embrio, dengan embrio globuler awal warna kuning cenderung lebih cepat berkembang ke stadia kotiledon dan kecambah dibandingkan dengan embrio hijau dan merah.

Summary

Somatic embryo culture of tea (*Camellia sinensis* L.) on an agar-solidified medium consists of embryos of different sizes, colors and developmental stages. One gram of mostly globular somatic embryos were cultured on a solid proliferation medium of WP containing 57.1 μM IAA and 4.4 μM BAP to observe their morphological variations with respect to embryo size, color, and developmental stage over one culture passage of 6 weeks. Fresh weight of somatic embryos increased slowly during the first 4 weeks and then sharply thereafter. At the fourth week, the number of embryos increased considerably although their weight did not increase, indicating the formation of secondary embryos. The average size of tea somatic embryos did not change significantly over the culture period, however, the embryo size was already highly varied at the start and increased as the embryo developed. About one half of the embryos were yellowish and the rest were divided equally between the greenish and reddish embryos. At the initial culture, 60% of the embryos were at the globular, 30% at heart and 10% at torpedo stage. Generally, globular embryos developed into later-stage embryos as the culture progressed, however, on this proliferation medium

almost 80% of the embryos remained at the globular and heart-shaped stages even after the sixth week. If single globular somatic embryos with a particular color were cultured on a solid regeneration medium of WP with 0.47 μM kinetin, 0.69 μM ABA and 0.29 μM GA₃, some of them especially the yellowish embryos underwent color change. Most of these single globular embryos developed gradually into the later stages. While the initial colors of embryos affected the rate of developmental stage changes, yellowish globular embryos tended to develop more rapidly into cotyledonary or germinant stages than the greenish and reddish embryos.

[*Keywords:* *Camellia sinensis*, embryo color, embryo development, somatic embryogenesis, tea]

Introduction

In vitro propagation of tea (*Camellia sinensis* [L.] O. Kuntze) has been achieved through proliferation of axillary buds (Nakamura, 1991; Agarwal *et al.*, 1992; Tahardi, 1994) and more recently through somatic embryogenesis (Wachira & Ogada, 1995; Ponsamuel *et al.*, 1996; Akula & Dodd, 1998; Akula *et al.*, 2000; Tahardi *et al.*, 2000). As a micropropagation system, somatic embryogenesis is considered superior due to its rapid multiplication rate, possibility for large-scale production in bioreactors, and generating embryos with well-developed taproots that are important for plant vigor and tolerance to drought in the field.

Somatic embryogenesis in tea has been induced from nodal or cotyledon explants generally without an intervening callus phase. Somatic embryos cultured on a solid medium

are usually present in all stages of development (globular, heart, torpedo and cotyledonary) at any one time because of repetitive (secondary) embryogenesis (Akula & Dodd, 1998; Akula *et al.*, 2000). Direct production of new embryos on the surface of previously differentiated embryos is very common in *Camellia* spp. (Vieitez & Barciela, 1990). In most of tea embryogenic systems, somatic embryo development did not require two phase culture procedures, the development and maturation of embryos occurred in the induction medium (Vieitez, 1995).

The morphology of somatic embryos of *Camellia japonica* was influenced by the concentration of cytokinin in the media (Vieitez & Barciela, 1990). In *C. sinensis*, Jha *et al.* (1992) identified three different types of somatic embryos: white 'seed-like' embryos, green 'cup-shape' embryos and globular embryos. However, the formation of these different types of embryos was not related to plant growth regulator in the media (Jha *et al.*, 1992). The morphological variations of somatic embryos limit the scaling up of *in vitro* mass propagation (Tautorus & Dunstan, 1995) and the development of synthetic seed technology (Attree & Fowke, 1993; Onishi *et al.*, 1994).

This experiment was conducted to determine the extent of morphological variations during tea somatic embryo development on agar-solidified media with respect to developmental stage, embryo size and color. An understanding of this phenomenon may help in improving the cultural procedure and conditions of tea somatic embryogenesis and in developing synthetic seed technology

Materials and Methods

Plant materials and culture conditions

Somatic embryos of tea clone TRI 2025 were produced from nodal cultures (Akula & Dodd, 1998) and subcultured regularly every 5 – 6 weeks on proliferation media. Embryos mostly at the globular stage with different colors were collected and used as experimental materials. All cultures were incubated in the culture room at $25 \pm 2^\circ\text{C}$ under cool-white fluorescent lamps providing approximately $30 \mu\text{mol photon/m}^2/\text{s}$ over a 14-h photoperiod.

Embryo composition

Mostly globular embryos weighing about 1 g in total were cultured on a 35 mL of proliferation medium in a 300 mL glass jar. The proliferation medium was a Woody Plant (WP) medium (Lloyd & McCown, 1981) with 30 g/L sucrose, 2 g/L Gelrite, 57.1 μM IAA and 4.4 μM BAP. Culture media were adjusted to pH 5.7 and autoclaved at 121°C and 1.0 kg/cm^2 for 20 min. A destructive method was used to determine somatic embryo development during 6 weeks of culture by harvesting 5 jars selected randomly every week. Growth and development were measured by embryo fresh weight and number. The length of each embryo was measured using a vernier caliper and its color and developmental stage were determined. Embryo heterogeneity was determined by embryo size and its coefficient of variance (CV). CV is defined as the sample standard deviation divided by the mean and expressed as percent (Steel & Torrie, 1980). In addition, morphological variations of somatic embryos

were also represented by different embryo colors and developmental stages at a specified time of culture.

Embryo development

Single globular embryos were collected and separated according to their colors (yellowish, greenish and reddish) and cultured on solid regeneration media. The regeneration medium was a WP medium with 30g/L sucrose, 2 g/L Gelrite, 0.47 μM kinetin, 0.69 μM ABA and 0.29 μM GA₃. As much as 35 mL of the medium was placed in a 10 cm-diameter Petri dish. Twenty single embryos with the same color were placed on the medium and replicated 8 times. The number of embryos was counted weekly for 8 weeks on the basis of color and developmental stages.

Results and Discussion

Embryo composition

Embryo growth

The fresh weight of somatic embryos increased slowly during the first 4 weeks in culture and then sharply thereafter (Figure 1). The fresh weight of embryos was almost three times of the initial weight by the end of experiment (6 weeks). The number of somatic embryos per jar increased in a similar pattern as the weight of embryos, except the exponential phase started earlier at the third week (Figure 1). The reason for this difference is perhaps due to the formation of secondary embryos at the fourth week that increased the number of embryos but did not affect the weight significantly because of their

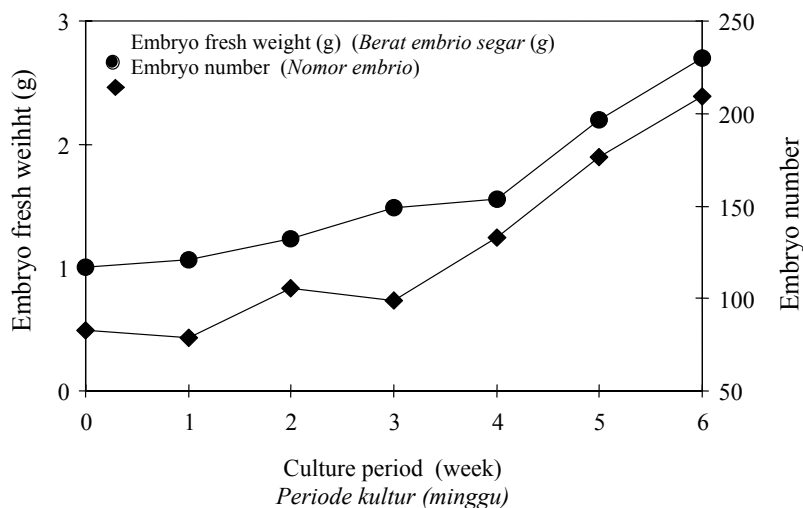


Figure 1. Fresh weight (●) and total number (μ) of tea somatic embryos per jar on a solid medium over a 6-week culture period.

Gambar 1. Berat segar (●) dan jumlah total (μ) somatik embrio teh per jar pada medium padat setelah 6 minggu dikulturkan

very small size. At week-6 there were more than 200 embryos covering almost entire medium surface in a jar from about 80 embryos at the beginning (Figure 1). This multiplication rate however, is lower than the results by Tahardi *et al.* (2000) who obtained a 2.6-fold increase within 4 weeks on a semi-solid half-strength MS medium, and by Akula & Dodd (1998) who obtained 4 – 8-fold within 6 weeks after culturing primary globular-stage embryos on MS basal medium with half-strength macro-salts and devoid of growth regulators. This difference could be attributed to differences in culture and environmental conditions.

Embryo size

The average size of tea somatic embryos at the initial culture was 2.52 mm (Table 1). The embryo size had increased slowly up to week 3 (2.89 mm) but dropped at week 4 (2.55 mm). This decrease may be due to secondary embryogenesis as indicated by the formation of many small globular embryos on primary embryos. Although the total fresh weight of somatic embryos at week 6 was almost triple of the initial weight (Figure 1), the average size of somatic embryos over one culture passage did not change significantly (Table 1).

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Table 1. Tea somatic embryo size and its coefficient of variance (CV) over one passage of culture.

Tabel 1. Ukuran dan koefisien varian (CV) somatik embrio teh setelah satu kali disubkultur

Culture period <i>Periode kultur</i> week (<i>minggu</i>)	Embryo size <i>Ukuran embrio</i> (mm)	CV of embryo size <i>CV dari ukuran embrio</i> (%)
0	2.52	40.6
1	2.51	42.5
2	2.62	42.9
3	2.89	54.4
4	2.55	55.1
5	2.71	58.3
6	2.55	60.3

The variation in embryo size as indicated by its coefficient of variation (CV) (Table 1) reveals that the size of somatic embryos was already highly varied at the start of the culture. A CV value higher than 30% indicates high variability. CVs of embryo size have increased steadily from 40 to 60% as the culture progressed to the sixth week (Table 1). At the end of the culture passage, many embryos have reached cotyledonary or germinant stages with the size longer than 5 mm, while many more new embryos at globular or heart stages with the size less than 2 mm were also present. Similar results obtained by Akula & Dodd (1998) revealed that the older embryos which increased in size up to 3-5 mm diameter grew into complete plantlets, while most of the other embryos demonstrated the tendency of repetitive embryogenesis by budding off new globular embryos. Therefore, all stages of developing embryos with different sizes were present at any one time over one passage of tea somatic embryo culture (Figure 2). On the other hand,

Wachira & Ogada (1995) did not find secondary embryogenesis in their tea somatic embryo cultures.

Embryo color

A group of tea somatic embryos on a solid medium were composed of different colors. At the start of the culture, about one-half of the embryos were yellowish and the rest were divided equally between greenish and reddish embryos (Figure 3). The percentage of the yellowish embryos decreased gradually up to week three, while the greenish embryos increased. The formation of secondary somatic embryos at week four is assumed to be responsible for the increase of yellowish embryos percentage where most of the new secondary embryos were yellowish in color. By the end of the experiment, the percentage of yellowish embryos decreased slightly, the percentage of greenish embryos was considerably stable, and the percentage of reddish embryos increased slightly (Figure 3).

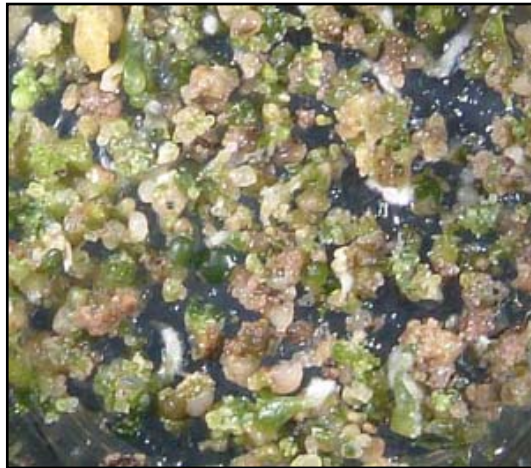


Figure 2. Somatic embryo culture of tea on a solid medium where different sizes, colors and developmental stages of embryos were found at the same time during the culture.

Gambar 2. Kultur somatik embrio teh pada medium padat dengan berbagai ukuran, warna dan fase perkembangan embrio yang ditemukan pada waktu yang sama selama dikulturkan.

Color differences among tea somatic embryos have not been mentioned by other research workers (Wachira & Ogada, 1995; Ponsamuel *et al.*, 1996; Akula & Dodd, 1998; Akula *et al.*, 2000), although Akula & Dodd (1998) reported that brownish trans-lucent cultures produced yellowish globular clumps of primary somatic embryos. Somatic embryo color has been used for quality evaluation of synthetic seeds of carrot (Sakamoto *et al.*, 1992) and for assessing somatic embryo conversion potential of sweet potato (Padmanabhan *et al.*, 1998).

Embryo developmental stage

At the initial culture, 60% of the embryos were at globular stage, 30% at heart stage and

10% at torpedo stage. During the first and second weeks, the globular-stage embryos had grown and changed to heart-stage embryos and the heart-stage embryos had grown to torpedo-stage embryos. This can be observed by the decrease of globular embryo number and the increase of heart and torpedo embryos (Figure 4). By the third week of culture, the number of globular embryos started to increase again, indicating early secondary embryo formation. At week four a larger proportion of the embryos was at the globular stage, confirming the occurrence of secondary embryogenesis. Secondary embryogenesis was also indicated by a sharp increase of small yellowish embryos (Figure 1 and 3).

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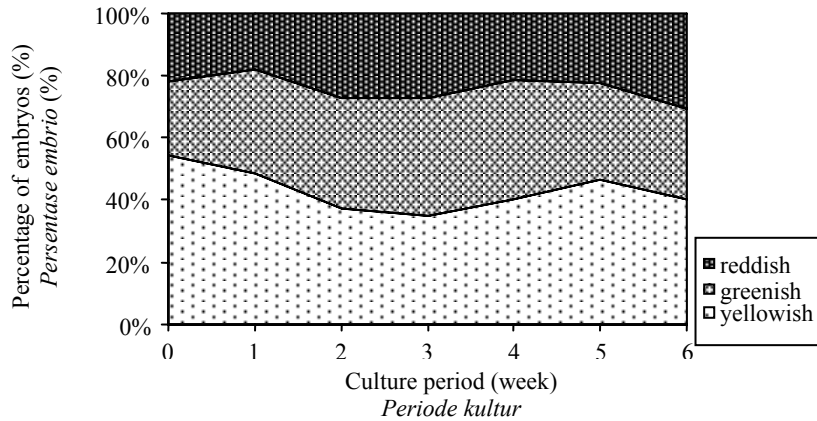


Figure 3. Color changes of tea somatic embryo composition over one passage of culture.

Gambar 3. Perubahan warna dan komposisi pasangan somatik embrio teh yang dikulturkan.

Germinant-stage embryos were already found at the third week; however by the end of the experiment at week six there were no plantlets with complete leaves and roots (embryo conversion). Akula & Dodd (1998) reported that 20% of the globular embryos converted into plantlets whereas 60% of the embryos formed secondary embryos. The formation of new globular embryos during the cultures and the development of certain stage of embryos into more advanced developmental stages result in the presence of developing embryos of all stages at any time over the culture period (Figure 2). This (highly asynchronous culture of tea somatic embryos was derived either from nodal culture (Akula & Dodd, 1998) or seed culture (Akula *et al.*, 2000). The heterogeneous characteristics of

tea culture on a solid medium can be reduced significantly by the use of a suspension culture (Tahardi *et al.*, 1997) or a temporary liquid immersion system (Tahardi *et al.*, 2000).

Embryo development

Single globular embryos with different colors were cultured separately on a solid regeneration medium to observe the changes in term of their colors and developmental stages over one passage of 8-week culture.

Color changes

More than half of the originally yellowish embryos turned to greenish and one-third to reddish color after they had been cultured on a solid regeneration medium for

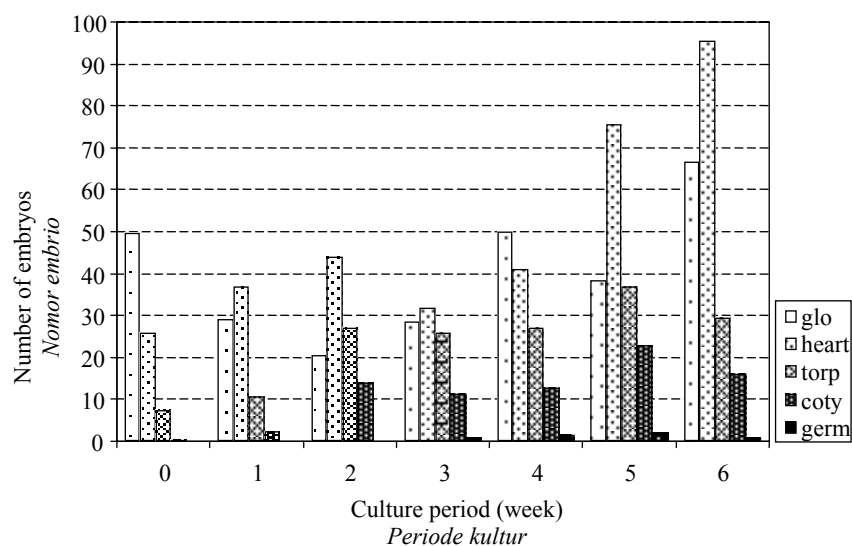


Figure 4. Number of different stages of tea somatic embryos over one passage of culture.

Gambar 4. Jumlah dari berbagai fase somatik embrio teh setelah satu kali disubkultur.

eight weeks (Table 2). In contrast, two-thirds of the originally greenish and reddish embryos remained unchanged in color. About one-quarter of the greenish embryos turned reddish and vice versa. By the end of the experiment, most of the embryos were either greenish or reddish and only less than 10% were yellowish. These results show that the color of somatic embryos may change during the culture on a solid medium. Most of the new globular embryos were yellowish as mentioned by Akula & Dodd (1998) but then turned greenish or reddish under light conditions. Changes in embryo color might be different if the cultures were placed in the dark.

Developmental stage changes

Single embryos with different colors were inoculated at the globular stage. These globular embryos developed gradually into more advanced developmental stages as the cultures progressed. One example of the pattern of developmental stage changes is taken from originally reddish embryos (Figure 5). The figure shows that the proportion of globular embryos reduced steadily while other stages started to appear. At the second week, the globular, heart and torpedo stages of embryos were already found. Cotyledonary stage of originally reddish embryos emerged at

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Table 2. The distribution of tea somatic embryos with respect to color after 8 weeks of culture on a solid WP medium.

Tabel 2. Distribusi warna pada somatik embrio teh setelah 8 minggu dikulturkan pada medium padat WP

Origin of embryo <i>Asal embrio</i>	Embryo color (<i>Warna embrio</i>) (%)		
	Yellowish <i>Kekuning-kuningan</i>	Greenish <i>Kehijau-hijauan</i>	Reddish <i>Kemerah-merahan</i>
Yellowish <i>Kekuning-kuningan</i>	6.6	60.0	33.4
Greenish <i>Kehijau-hijauan</i>	7.5	65.9	26.6
Reddish <i>Kemerah-merahan</i>	10.9	23.5	65.6

the third week, whereas germinant stage appeared at the fourth week. These results indicate that single somatic embryos are changed into later developmental stages gradually, not at the same time for all embryos. For example only about 10% of the embryos were at cotyledonary stage at the fourth week and increased to 20% by the end of experiment. Asynchronous development of these somatic embryos may be due to differences in embryo sources and culture conditions. Single globular somatic embryos used might not exactly the same in terms of size and developmental stage. In addition, only a part of embryo surface was connected to the surface of solid medium. However, even in suspension cultures where all surface of cells or embryos are exposed to the liquid medium, asynchronous development of somatic embryos of woody species is still very common (Taurus & Dunstan, 1995). At the end of the experiment, almost three-quarters of the originally yellowish globular embryos were at the torpedo, cotyledonary or germinant stages (Table 3). Only 3% of the embryos

remained at the globular stage. The originally greenish embryos developed slower than yellowish embryos, only 10% were at germinant stage and 11% remained at globular stage. Almost half of the originally reddish embryos were still at the globular or heart stages after 8 weeks of culture, while about one third of the embryos were at the cotyledonary or germinant stages (Table 3). These results show that not all of the globular embryos were converted into the later stages and the initial embryo colors affected the rate of embryo stage changes during the culture on a solid medium. Initial yellowish globular embryo changed more rapidly into cotyledonary or germinant stages (almost 50%) than those of greenish and reddish embryos (33-34%) (Table 3). Different colors of tea somatic embryos may have different levels of storage reserves such as starch and triacylglycerol that were found to be responsible for the germination of somatic embryos into plantlets in *Hevea* rubber (Cailloux *et al.*, 1996).

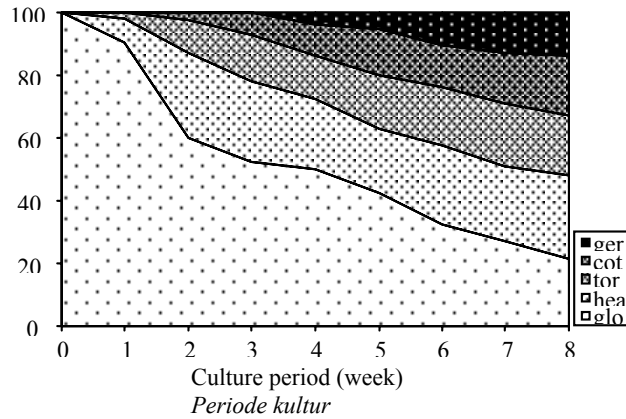


Figure 5. Developmental stage changes of originally reddish globular embryos over 8 weeks of culture on a regeneration solid medium.

Gambar 5. Perubahan fase perkembangan embrio yang berasal dari globular setelah 8 minggu dikulturkan dalam medium padat untuk regenerasi.

Table 3. The distribution of tea somatic embryo with respect to developmental stage after 8 weeks of culture on a solid WP medium.

Tabel 3. Distribusi somatik embrio tehe berdasarkan tingkat perkembangan setelah 8 minggu dikulturkan pada medium WP yang padat.

Origin of embryo <i>Asal embrio</i>	Embryo developmental stage (%) <i>Tahap perkembangan embrio (%)</i>				
	Globular <i>Globular</i>	Heart <i>Hati</i>	Torpedo <i>Torpedo</i>	Cotyledonary <i>Kotiledon</i>	Germinant <i>Kecambah</i>
Yellowish <i>Kekuning-kuningan</i>	3.1 b*	20.0 b	27.1 a	25.1 a	24.6 a
Greenish <i>Kehijau-hijauan</i>	11.4 b	32.3 a	22.1 ab	24.6 a	9.6 b
Reddish <i>Kemerah-merahan</i>	21.5 a	27.1 ab	18.5 b	19.0 a	14.0 b

*Means in the same column followed by the same letters are not significantly different at P=0.05 according to Duncan's multiple range test.

* Angka pada kolom yang sama diikuti dengan huruf yang sama tidak berbeda nyata P=0,05 menurut uji jarak berganda Duncan.

In developing synthetic seed production, the availability of somatic embryos at the developmentally mature stages (cotyledonary and germinant) is important as material sources. The experimental results show that higher numbers of originally yellowish embryos had been converted into later developmental stages than those of greenish and reddish embryos. It means that yellowish embryos are better sources of materials for synthetic seeds. In addition, the germination rate of somatic embryo is also essential to obtain high conversion frequency of synthetic seeds to plants (Redenbaugh *et al.*, 1987). The high variability of tea somatic embryos will restrain the development of mass clonal production and synthetic seed technology of tea. Therefore, attempts to synchronize somatic embryo development of tea have been conducted by using a suspension culture (Tahardi *et al.*, 1997; 2000).

Conclusions

Tea somatic embryos on a solid medium was usually composed of different sizes, colors, and developmental stages at any time during the culture. The average size of tea somatic embryos did not change considerably over one passage of culture, however their size variability increased as the culture progressed. At the initial culture, 50% of the embryos were yellowish, 25% greenish and 25% reddish whereas at the sixth week of culture the proportion of yellowish embryos decreased slightly. The developmental stage of the embryos was changed over the course of culture. Globular embryos of originally yellowish color developed more rapidly into

cotyledonary or germinant stages than the greenish and reddish embryos, therefore more suitable as material sources for synthetic seed production

Acknowledgment

This research was supported by a grant from the Australian Centre for International Agricultural Research (ACIAR), Canberra, Australia.

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