INDUCTION OF SOMATIC EMBRYOS IN CULTURED LEAF EXPLANTS OF *Coffea arabica.*

Oleh : *E. Noerhadi*, T. Yasuda**, Arbayah Siregar*, and Risco G. Budji****

SARI

Tulisan ini menguraikan induksi embrio somatik dan pertumbuhan planlet yang dihasilkan dari kultur jaringan daun *Coffea arabica.* Untuk pembentukan kalus, potongan jaringan daun ditanam pada medium Linsmaier dan Skoog dengan 3% sukrosa ditambah berbagai konsentrasi 2,4-asam diklorofenoksi asetat atau asam naftalen asetat dan kinetin. Delapan belas minggu setelah penanaman, kalus akan dibentuk pada medium Linsmaier dan Skoog dengan 2 μM 2,4-asam diklorofenoksi asetat dan 5–7 μM kinetin dan juga pada medium dengan 0,05 μM asam naftalen asetat dan 6–8 μM kinetin. Pembentukan embrio somatik diperoleh dengan pemotongan kalus pada medium padat Linsmaier dan Skoog dengan 3 μM sampai 35 μM kinetin dan 0,05 μM asam indole butirat dan juga dalam medium cair Gamborg, Miller, dan Ojima yang diberi 0,05 μM sampai 2,5 μM 2,4-asam diklorofenoksi asetat.

ABSTRACT

Somatic embryo induction and subsequent plantlets development in culture of *Coffea arabica* leaf tissue explants was described. For callus formation leaf segments were grown on medium Linsmaier and Skoog with 3% sucrose and varying concentrations of 2,4-Dichlorophenoxyacetic acid or Naphthalene Acetic Acid and Kinetin. Eighteen weeks after inoculation, callus will be formed on Linsmaier and Skoog's medium with 2 μM 2,4-Dichlorophenoxyacetic acid and 5–7 μM Kinetin and also on medium with 0.05 μM Naphtalene Acetic Acid and 6–8 μM Kinetin. Somatic embryos are then formed by inoculation of calli segments on medium Linsmaier & Skoog with 3 μM to 35 μM Kinetin and 0.05 μM Indole Butyric Acid. Somatic embryos are also formed in liquid medium of Gamborg, Miller and Ojima supplemented with 0.05 μM to 2.5 μM 2,4-Dichlorophenoxyacetic acid.

* Department of Biology, Institut Teknologi Bandung , Indonesia
** Department of Agriculture and Horticulture, Kobe University, Japan.
*** Department of Biology, Universitas Hasanuddin, Indonesia.

Introduction

Coffee is one of the most important agriculture commodities in the international market. Its production is restricted to tropical countries including Indonesia. There are two commercially important coffee species: *Coffea arabica* L. (Arabica coffee) and *C. canephora* Pierre (Robusta coffee). Quality beverage is produced from Arabica coffee which represent 70% of the commercial coffee of the world (Sondahl *et al.*, 1985). Those two important species are propagated via seeds. Propagation using tissue culture might provide a superior alternative to seed or vegetative propagation by cuttings. Recent advances of cell and tissue culture open the possibility of applying the improvement of economically important plants (Smith, 1974; Chaleff and Carlson, 1974). Coffee geneticists are interested in the application of this technology for the regeneration and selection of genetic variability for obtaining new cultivars.

Staritsky (1970) published the first work on coffee tissue culture in which callus cells were successfully cultivated using explants from soft internodes of young orthotropic shoots of *Coffea arabica*, *C. canephora*, and *C. liberica*. He also succeeded in producing somatic embryos and plantlets of *C. canephora* shoots. For these studies Staritsky used a modified medium of Linsmaier & Skoog with 2,4-Dichlorophenoxyacetic acid (2,4-D) 0.1 mg/l or Naphthalene Acetic Acid (NAA) 1.0 mg/l and Kinetin 0.1 mg/l. Herman and Haas (1975) have reported of obtaining organoids from leaf explants of *C. arabica* cultured on medium Linsmaier & Skoog (medium LS) supplemented with 0.46 μM Kinetin and 0.45 μM 2,4-D after 60 days in darkness. Many of these organoids showed abnormal leaf formation. Selected organoids characterized by green spots with normal leaves were subcultured onto medium Gresshoff and Doy no. 4 containing 0.54 μM NAA in the absence of Kinetin. Root formation occurred after 60 days and following 7 months the surviving plantlets were transferred to soil. Recently, Sondahl *et al.* (1985) obtained plantlets of coffee by somatic embryogenesis and auxillary bud culture. Leaf explants were grown on Murashige & Skoog's inorganic salts, 30 μM thiamine-HCl, 210 μM L-lysine, 550 μM meso-inositol, 117 μM sucrose, and 8 g/l Difco Bacto agar supplemented with 20 μM Kinetin and 5 μM 2,4-D. Somatic embryogenesis was observed on Murashige & Skoog's organic salts, 58.4 μM sucrose, 2.5 μM Kinetin, and 0,5 μM NAA. In these studies of somatic embryogenesis, plant regeneration with coffee tissue has been accomplished from solid cultures using relatively high amount of 2,4-D or NAA.

In our laboratory at the Dept. of Biology, ITB, attempts have been made to evaluate the feasibility of developing various aseptic techniques for clonal multiplication and improvement of *C. arabica*. This paper reports the results of initial experiments designed to establish somatic embryogenesis using solid

and liquid cultures with relatively high amounts of Kinetin in the absence or with relatively low amount of NAA.

Materials and Methods

The original coffee plants were cultivated in the area of Pengalengan near Cibiana at an altitude of about 900 meter above sea level and obtained through the Perkebunan Rakyat Tanaman Pangan Ekspor in West Java. Young leaves from plagiotropic branches of about 2 years old *C. arabica* plants were surface sterilized in 1.6% Na-hypochlorite with 0.1% Tween 20 as surfactant for 20 minutes, then washed several times in sterile distilled water. Interveinal segments about 5 mm^2 were excised and transferred to basal medium for callus formation. The source of experimental material was callus obtained from these leaf segments. Therefore, some parts of the callus were inoculated on solid medium of Linsmaier & Skoog (1965) for multiplication of callus and for induction of somatic embryogenesis. Some other parts of the callus were transferred into liquid medium of Gamborg, Miller, and Ojima (1968) for somatic embryogenesis. All the cultures were grown in diurnal condition at room temperature.

The basal nutrient medium for all callus cultures is of Linsmaier and Skoog (1965) or LS medium containing the following substances (mg/liter): NH_4NO_3 1650; KNO_3 1900; $CaCl_2.2H_2O$ 440; $MgSO_4.7H_2O$ 370; KH_2PO_4 170: Na_2 EDTA 37.3; $FeSO_4.7H_2O$ 27.8; H_3BO_3 6.2; $MnSO_4.7H_2O$ 22.3; $ZnSO_4.7H_2O$ 8.6; KI 0.83; $Na_2MoO_4.2H_2O$ 0.25; $CuSO_4.5H_2O$ 0.025; $CoCl_2.6H_2O$ 0.025; thiamine-HCl 0.8; myo-inositol 100; sucrose 30 g/l; and agar 8 g/l. The pH is adjusted to 5.7 before sterilization in an autoclave at 120°C for 15 minutes. The basal nutrient medium for liquid culture is of Gamborg, Miller, and Ojima (1968) or GA medium which contains the following substances (mg/liter): KNO_3 2500; $CaCl_2.2H_2O$ 150; $MgSO_4.7H_2O$ 250; $(NH_4)_2SO_4$ 134; $NaH_2PO_4.H_2O$ 150; KI 0.75; H_3BO_3 3.0; $MnSO_4.H_2O$ 10; $ZnSO_4.7H_2O$ 2.0; $Na_2MoO_4.2H_2O$ 0.25; $CuSO_4.5H_2O$ 0.025; $CoCl_2.6H_2O$ 0.025; Na_2EDTA 37.3; $FeSO_4.7H_2O$ 27.8; myo-inositol 100; thiamine-HCl 10; pyridoxine-HCl 1.0; nicotinic acid 1.0; sucrose 20 g/l; and pH 5.7.

Results and Discussion

Culture of leaf segments. Two auxins 2,4-Dichlorophenoxyacetic acid (2,4-D) or Naphthalene Acetic Acid (NAA) and one cytokinin in the form of Kinetin were used for callus initiation on leaf segments of *Coffea arabica*. For this purpose different combination and concentrations of growth regulators were used. In cultures on LS medium with 2 μM 2,4-D and 5–7.5 μM Kinetin organized growth occured 18 weeks after inoculation (Fig. 1 A). In cultures contain-

ing 0.5 μM NAA compact callus were formed after two weeks. However these calli soon became brown and failed to be subcultured. The only organized growth occured on LS medium containing 0 or 0.05 μM NAA and 6–8 μM Kinetin. White and friable callus were formed. Globular tissues and "green spots" as signs of shoot formation appeared on the surface of the callus (Fig. 1 B). Somatic embryos will then be formed 5 weeks after callus initiations (Fig.2).



Fig. 1 A Callus formation on leaf segments grown on medium Linsmaier and Skoog with 2 μM 2,4-D and 5–7,5 μM Kinetin.

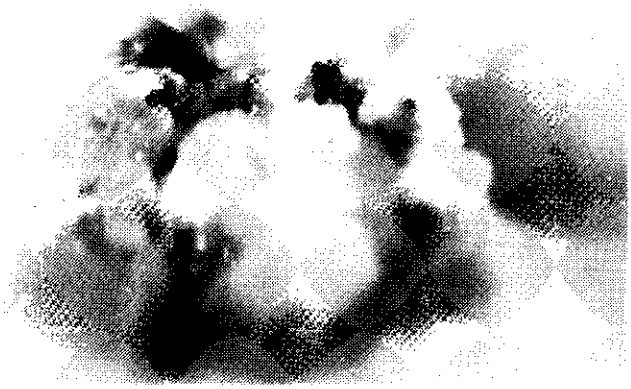


Fig. 1 B Close-up view on globular types and "green spots" on the surface of the callus.

From the above results it is again clear that for callus formation both auxin and cytokinin are necessary. However, the amount of the respective growth regulators might be different. For example, Staritsky (1970) and Herman & Haas (1975) or Sondahl and Sharp (1975, 1977) and Sondahl *et al.* (1985) needed relatively high amount of auxin and relatively low cytokinin for the induction of callus on leaf segments of *C. arabica* and for their induction of somatic embryos. In contrast we have found for this inductions relatively low amount of auxin and relatively high amount of cytokinin. The same phenomena of direct somatic embryogenesis were observed by Dublin (1981) and Yasuda *et al.* (in press). However, Dublin (1981) used medium of Murashige & Skoog (1962) containing high content of cytokinin in the form of Benzylaminopurine (BAP). He studied leaf segments of *Arabusta* coffee and needed 0.01–1 mg/l 2,4-D and 1–10 mg/l BAP for somatic embryogenesis. Yasuda *et al.* (in press) studied somatic embryogenesis of *C. arabica* on medium Murashige and Skoog containing only 5 μM BAP.

Culture of leaf callus. Leaf calli were subcultured on two different kinds of media: a solid medium of LS and a liquid medium of GA. Callus which was formed on LS medium containing 0 or 0.5 μM NAA and 6–8 μM Kinetin was subcultured on a similar medium for multiplication and somatic embryogenesis. However, plantlets were subcultured on LS medium without growth regulators (Fig. 3) or LS medium with Indole Butyric Acid (IBA) as much as 0.05 μM for root formation.

Callus segments originating from LS media containing 2,4-D and Kinetin were all transferred into GA liquid medium with 0–0.25 μM 2,4-D. Organized growth was observed 8–10 weeks after transferring. With varying length of time in culture random cell division actively occured. These scattered regions of high mitotic activity led to the formation of aggregates, which became more friable and continued to divide. Continue division of the surface led to the formation of small proturbances giving the tissue a nodular appearance which then produced embryos. These embryos were released when they reached the globular stage (Thorpe, 1982; Sondahl *et al.*, 1985). This stage was followed by heart stage and torpedo stage (Fig. 4 A, B, C) and cotyledonary stage. At a later stage these embryos were subcultured from the liquid medium (Fig 5 A) to GA solid medium containing 0.5% sucrose and 0–0.1 μM 2,4-D. Within 4–6 weeks after transferring these somatic embryos developed into plantlets (Fig. 5 B). This pattern of *in vitro* embryogenic development could be considered as Induced-Embryogenic Determined Cells (IEDC) as proposed by Sondahl and Sharp (1977).



Fig. 2 Spongy type of callus tissue (left), somatic embryos, and plantlets (right) of *Coffea arabica*.



Fig. 3 Older plantlets grown on LS medium with 3% sucrose without growth regulators.

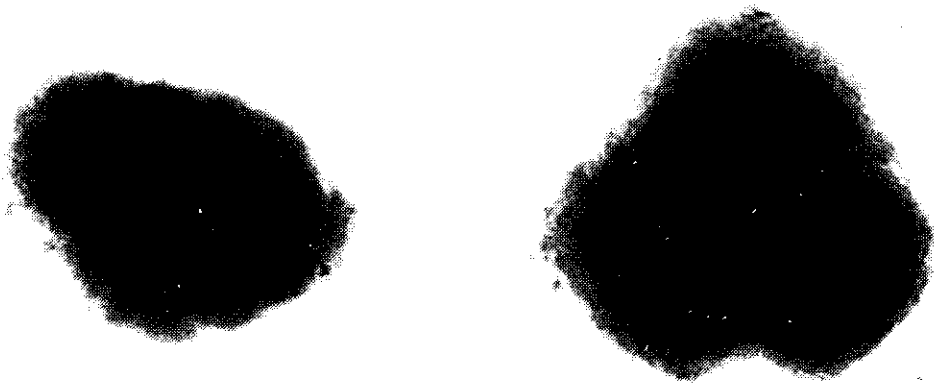


Fig. 4 A and B Globular and heart stage embryos.

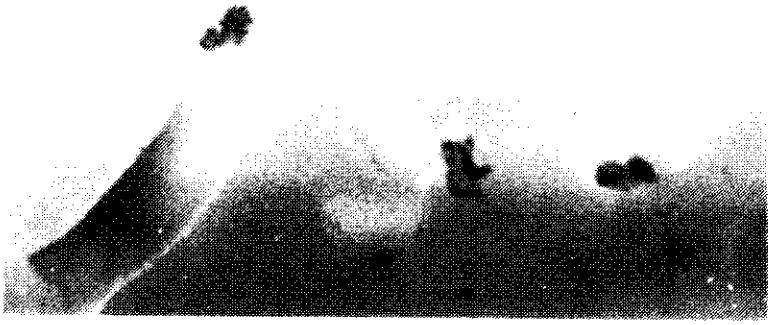


Fig. 4 C Terpedo stage embryos.



Fig. 5 A Somatic embryos formed in liquid culture.



Fig. 5 B Plantlets grown on GB medium (see text).

Acknowledgement

The research reported and discussed in this paper is one of the results of cooperative research programme between the Japan Society for the Promotion of Science (JSPS) in Tokyo and the Directorate General of Higher Education (DGHE) in Jakarta. To all those institutes we all are thankful and grateful.

References

Chaleff, R. S. and P. S. Carlson (1974), Somatic cell genetics of higher plants, *Ann. Rev. Genet., 8*: 267–277.

Dublin, P., (1981). Embryogenèse somatique directe sur fragments du fuilles de cafeier *arabusta, Cafe Cacao The, 25*: 237–241.

Evans, D. A., W. R. Sharp, and C. E. Flick (1981). Growth and behaviour of cell culture: Embryogenesis and organogenesis; In: *Plant tissue culture, Methods and application in agriculture*, T. A. Thorpe, Acad. Press, New York, *p.* 45–114.

Gamborg, O. L., R. A. Miller, and K. Ojima (1968). Nutrient requirements of suspension culture of soybean root cells, *Exp. Cell Res., 50*: 151–158.

Herman, E. B. and G. J. Haas (1975). Clonal propagation of *C. arabica* L. from callus culture. *Hort. Sci., 10*: 588–589.

Linsmaier, E. M. and F. Skoog (1965). Organic growth factor requirements of tobacco tissue culture, *Physiol. Plant., 18*: 100–127.

Murashige, T. and F. Skoog (1962). A revised medium for rapid growth on bio-assay with tobacco tissue culture, *Physiol. Plant., 15*: 473–479.

Smith, H. H., (1974). Model systems for somatic cell plant genetics, *Bio-Science, 24*: 267–276.

Sondahl, M. R. and W. R. Sharp (1975). The growth of *Coffea arabica* leaf cells in culture, XII Interntl Botan. Congress, P. 317, Leningrad.

Ibid, (1977). High frequency induction of somatic embryos in cultured leaf explants of *Coffea arabica* L. Z., *Pflanzenphysiol., 81*: 395–408.

Sondahl, M. R., T. Nakamura, and W. R. Sharp (1985). Propagation of coffee. In: *Tissue Culture in forestry and agriculture* Eds.: R. H. Henke, K. W. Hughes, M. J. Constantin, and A. Hollaender, Plenum Press, New York, *p.* 251–232.

Staritsky, G., (1970). Embryoid formation in callus tissue of coffee, *Acta Bot. Neerl., 19*: 509–514.

Thorpe, T. A., (1982). Callus organization and *de novo* formation of shoots, roots and embryos *in vitro*. In: *Application of plant cell and tissue culture to agriculture and industry,* Eds. D. T. Tomes, B. E. Ellis, P. M. Harney, K. J. Kasha, R. L. Peterson, University of Guelph, Guelph, Ontario, Canada. *p.* 115–138.

Yasuda, T.; Fujii, Y and T. Yamaguchi (1985). Embryonic callus induction from *Coffea arabica* leaf explants by Benzyladenine (in press).