THE EFFECT OF 2,4-D AND 2-iP ON CALLUS PROLIFERATION AND DEVELOPMENT ON IMMATURE LEAF EXPLANTS OF LIBERICA COFFEE (*Coffea liberica* L.)

Lizawati LIZAWATI[*], Zulkarnain ZULKARNAIN[*], Neliyati NELIYATI[*]

[*] *University of Jambi, Faculty of Agriculture, Department of Agroecotechnology, Kampus Pinang Masak, Jambi, Indonesia 36361*
Correspondence author: Zulkarnain Zulkarnain, University of Jambi, Faculty of Agriculture, Department of Agroecotechnology, Kampus Pinang Masak, Jambi, Indonesia 36361, email: dr.zulkarnain@yahoo.com

Abstract. An *in vitro* protocol for callus proliferation and development of *Coffea liberica* was developed. Immature leaf sections (1 x 1 cm) were cultured on solid MS medium supplemented with 2,4-D (0, 0.5, 1.0 and 2.0 mg L^{-1}) and 2-iP (0, 1.0, 2.0 and 3.0 mg L^{-1}). Cultures were maintained at temperature of 25 ± 1 °C, light intensity of 50 µmol m^{-2} s^{-1} and 16-hours daily photoperiod for 6 months. Callus growth and characteristics were evaluated for 6 months of culture. It was found that the application of 0.5 mg L^{-1} 2.4-D + 2.0 mg L^{-1} 2-iP resulted in the best time for callus induction (13.47 days after culture initiation). The application of 0.5 mg L^{-1} 2.D + 2.0 mg L^{-1} 2-iP and 1.0 mg L^{-1} 2.4-D + 1.0 mg L^{-1} 2-iP resulted in the highest frequency of explant forming callus (56.67% on the average). The morphology of proliferated callus varied from clear, white-creamy, light yellow and brown in color, and friable to compact in structures.

Keywords: *Coffea liberica*; tissue culture; micropropagation; 2,4-Dichlorophenoxyacetic acid; N^6-(2-Isopentenyl)adenine.

INTRODUCTION

Coffea is a member of the Rubiaceae family, whose seeds, are used for various coffee beverages and products. This plant is native to tropical and southern Africa and tropical Asia. There are at least 130 coffea genera have been recorded so far Campos et al. [6], and three of them are known as major genera within the familiy: *C. arabica, C. canephora* and *C. liberica.*

Coffea liberica is quite popular among farmers in the Tanjung Jabung Barat Regency of Jambi Province, Indonesia, and is known as Liberika Tungkal Komposit (Libtukom) clone. The propagation of Libtukom is normally by a generative method using seeds collected from field grown plants. The weakness of this method is that the nature of the progenies produced is less uniform and the time from planting to fruiting is relatively long. On the other hand, methods of conventional vegetative propagation through cutting or grafting are hampered by the limited amount of stock plant material and are seasonally dependent.

Another method of Libtukom propagation is through micropropagation. This technique could provide a viable alternative to conventional methods of propagation and offers massive scale and uniform progenies in a relatively short time [27]. More importantly, as the initial plant materials are from somatic tissues, the progenies derived from micropropagation will have similar important parental traits.

Coffea propagation through tissue culture has been successfully made for *arabica* and *canephora* species [1, 6, 7, 9, 13, 14, 21]. A study conducted by the Indonesian Agency for Agricultural Research and Development since 2012 showed that fully-opened immature leaves were appropriate plant materials for induction of tissue culture of *C. arabica* [11]. The effective use of young leaves as explant materials for induction of *C. arabica* tissue culture was also reported [1, 2]. In *C. liberica,* however, this is among few reports on tissue culture induction according to our knowledge. The effects of various concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D) and N^6-(2-Isopentenyl)adenine (2iP) on callus formation and proliferation on immature leaf explants of *C. liberica* were studied.

MATERIALS AND METHODS

Explant materials used were immature leaf sections of *C. liberica* clone Libtukom. The stock plants originated from field-grown individuals cultivated in Parit Tomo Village, Tanjung Jabung Barat Regency, Jambi Province, Indonesia.

Murashige and Skoog (1962) (MS) [16] medium supplemented with 20 g L^{-1} sucrose, vitamins, and solidified with 2 g L^{-1} gelrite was used. The pH was 5.7 and the medium was autoclaved at 121 °C for 20 minutes.

The plant growth regulators tested were 2,4-D (0, 0.5, 1.0 and 2.0 mg L^{-1}) and 2-iP (0, 1.0: 2.0 and 3.0 mg L^{-1}). Thus, there are 16 treatment combinations with 3 replicates. Each replicate consisted of 10 culture flasks containing one explant each.

Immature leaves were isolated from stock plants in greenhouse, washed thoroughly with 100 mL of sterile distilled water with four drops of Tween-20. After rinsing with sterile water, the leaves were soaked in 0.2% Dithane M-45 solution for 20 minutes, followed by soaking in 0.1% NaOCl solution for 20 minutes before being rinsed three times with sterile distilled water.

Leaf segments of approximately 1 cm^2, excluding midvein, leaf margins, and apical and basal parts were cultured on the above mentioned medium (one segment/flask). Cultures were kept in a culture room at 25 ± 1 °C, light intensity of 50 µmol m^{-2} s^{-1} and 16-hours daily photoperiod for 6 months. Subcultures were made every 8 weeks onto fresh medium with the same composition and growth regulators supplement. The process of callus formation was directly observed from the beginning of planting, and data were recorded on a weekly basis. The evaluated variables were callus induction time from culture initiation, the percentage of

explants forming callus, and the morphology of proliferated callus.

RESULTS

Callus proliferation was successfully induced from immature leaf explants of *C. liberica* cultured on medium with 2,4-D and 2-iP. The presence of 2,4-D along with 2-iP in culture medium promoted callus proliferation. On the other hand, the absence of 2,4-D and/or 2-iP resulted in the failure of callus formation.

The initial response of explants was the formation of swollen structures at the edges of cut section. Callus growth was first seen on the surface of explants within two weeks after culture initiation, and two weeks later callus was clearly formed on the surface of explants, except those cultured in medium without growth regulators or medium treated with 1.0 mg L^{-1} 2-iP alone (Table 1).

The color of callus formed on the surface of the explants varied from clear brown, white-creamy, light yellow, greenish yellow and dark brown, with friable or compact structure (Table 2).

DISCUSSION

The application 0.5 mg L^{-1} 2,4-D combined with 2-iP at 2.0 mg L^{-1} gave the best results for callus proliferation rate (13.47 and 14.83 days after culture, respectively). The combination of 2,4-D and 2-iP also enhanced the number of explant forming callus. The highest number of callusing explants (56.67%) was on culture medium with 0.5 mg L^{-1} 2,4-D + 2.0 mg L^{-1} 2-iP or 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 2-iP. In addition, the application of 1.0 mg L^{-1} 2,4-D without 2-iP lead to high percentage of explants forming callus (53.33%). This suggests that the presence of 2,4-D in culture medium is important to stimulate callus proliferation on immature leaf explants of *C. liberica*. This is in accordance with the finding of Rahman et al. [18] on rubber tissue culture, where the application of 1.0 – 2.0 mg L^{-1} 2,4-D produced callus. When the concentration of 2,4-D was increased to 2.0 mg L^{-1}, however, it was important to add 2.0 mg L^{-1} 2-iP in culture medium in order to produce high callus proliferation (50.00% of cultured explants).

Table 1. The effect of 2.4-D + 2-iP on callus proliferation on immature leaf explants of *C. liberica*

Growth regulators		Callus formation	
2.4-D (mg L^{-1})	2-iP (mg L^{-1})	Induction time (number of days after culture initiation ± SE))	Percentage of callusing explants (% ± SE)
0.0	0.0	-	-
	1.0	-	-
	2.0	16.83 ± 2.17	13.33 ± 10.18
	3.0	17.00 ± 7.00	6.67 ± 4.44
0.5	0.0	17.00 ± 0.00	3.33 ± 3.33
	1.0	14.92 ± 2.07	30.00 ± 13.56
	2.0	13.47 ± 0.96	56.67 ± 15.75
	3.0	20.00 ± 3.47	26.67 ± 12.96
1.0	0.0	19.67 ± 1.44	53.33 ± 15.07
	1.0	18.28 ± 0.99	56.67 ± 8.68
	2.0	14.83 ± 1.83	20.00 ± 13.33
	3.0	18.43 ± 1.96	43.33 ± 14.95
2.0	0.0	18.50 ± 2.29	13.33 ± 7.37
	1.0	17.58 ± 1.92	16.67 ± 11.39
	2.0	23.13 ± 2.05	50.00 ± 8.96
	3.0	23.00 ± 1.00	16.67 ± 11.39

Table 2. The effect of 2.4-D + 2-iP on the characteristics of callus formed on immature leaf explants of *C. liberica*

Growth regulators		Charateristics of callus	
2.4-D (mg L^{-1})	2-iP (mg L^{-1})	Callus color	Callus structure
0.0	0.0	-	-
	1.0	-	-
	2.0	Brownish cream	Compact
	3.0	Clear, white, cream	Compact
0.5	0.0	Brownish cream	Compact
	1.0	White, cream, light yellow	Friable to compact
	2.0	Clear, white, cream, light yellow	Compact
	3.0	Clear, white cream, light yellow	Friable to compact
1.0	0.0	White.cream, light yellow	Compact
	1.0	White.cream, light yellow	Compact
	2.0	Brownish cream	Compact
	3.0	Light yellow, cream, brown	Compact
2.0	0.0	Light yellow, cream,	Compact
	1.0	White, cream, light yellow	Compact
	2.0	Clear, white, cream, brown	Compact
	3.0	Clear, white, cream	Friable to compact

The difference of *in vitro* response showed by immature leaf explants of *C. liberica* is believed due to the difference in the balance of the 2,4-D and 2-iP in culture medium. As a comparison, in *C. arabica* tissue culture, Riyadi and Tirtoboma [19] reported that the application of 4.0 mg L^{-1} 2,4-D + 0.1 mg L^{-1} kinetin resulted in massive callus formation following 10 days after culture initiation. Callus started to proliferate from the wounded edge of explants, and proembryos were clearly identified two weeks later.

The color of callus proliferated on the explants surface in tissue culture is a visual indicator indicating that cells are actively dividing and growing or have already died. White, cream or yellowish callus indicates cells are growing, while brown callus indicates decreasing in growth. In this study, the color of callus was dominated by white-creamy, light yellow and yellowish green in color, and friable to mostly compact in structure. These properties indicate the general characteristics of embryogenic callus in *C. arabica* [6, 8, 17, 22]. The appearance of yellow embryogenic callus were not only found on coffee, but also on other species such as *Jatropha curcas* [25], sugarcane [5], and rubber [18].

In addition to color, its structure was also used to assess the embryogenic capacity of a callus clumps. Visual observations on calluses formed on the surface of immature leaf explants of *C. liberica* exhibited friable to compact structure (Table 2). This was in accordance with the findings of Ardiyani [2] in tissue culture of *C. liberica* clone Arruminensis. Friable callus was produced on explants treated with 2.0 mg L^{-1} 2,4-D + 3.0 mg L^{-1} 2-iP, 0.5 mg L^{-1} 2,4-D + 3.0 mg L^{-1} 2-iP, 0.5 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 2-iP, and 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 2-iP (Figure 1). Zulkarnain [26], Tapingkae et al. [23] and Kartika et al. [12] suggested that the structure of callus proliferating on the surface of explants may vary from friable to compact. Plant genotypes, type of explants, medium composition, growth regulators, environmental conditions of culture, and physiological age had a significant effect on embryogenic callus formation [23, 24, 26]. Friable structure was also reported by Romeida et al. [20] in embryogenic callus of *Papilionanthe hookeriana.* Bartos et al. [3] and Muniswamy et al. [15] reported embryogenic callus of *C. arabica* was characterized by a friable structure of intense yellowish color. In leaf explants of *Lachenalia montana* cultured on MS liquid medium supplemented with 0.23 mg L^{-1} 2,4-D the structure of embryogenic callus was friable [4]. In *Zingiber officinale,* Guo and Zhang [10] claimed that the 2,4-D shock could serve as a trigger to induce cell division in the epidermal cells and promote their further differentiation to somatic embryos. On maize, Zhong et al. [24] reported that the optimum 2,4-D concentration for the initiation of embryogenic callus varied from 2.0 mg L^{-1} to 3.0 mg L^{-1}.

This work was among few contributions in the efforts of establishing modern clonal propagation of

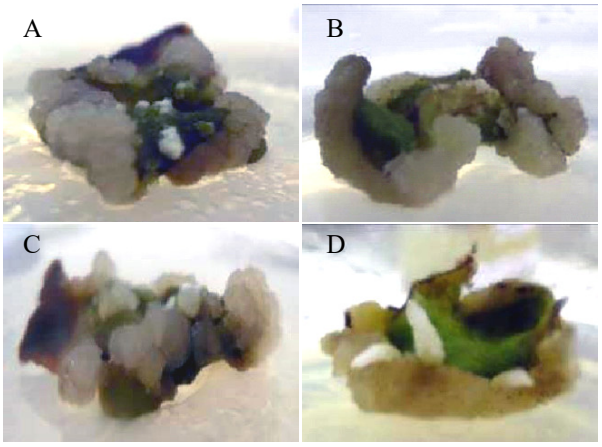

Figure 1. Morphology of callus proliferated on immature leaf explants of *C. liberica.* A = 2.0 mg L^{-1} 2,4-D + 3.0 mg L^{-1} 2-iP, B = 0.5 mg L^{-1} 2,4-D + 3.0 mg L^{-1} 2-iP, C = 0.5 mg L^{-1} 2,4-D + 2.0 mg L^{-1} 2-iP, D = 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 2-iP.

Coffea liberica we know so far. The significant findings of this investigation were:

1. The presence of 2,4-D along with 2-iP in culture medium undoubtedly promoted callus proliferation. The absence of 2,4-D and/or 2-iP in the medium resulted in no callus formation. The application of 1.0 mg L^{-1} 2,4-D + 1.0 mg L^{-1} 2-iP and 0.5 mg L^{-1} 2.4-D + 2.0 mg L^{-1} 2-iP were the best combination for inducing callus proliferation.
2. The proliferated callus was believed to have the opportunity to develop embryogenic properties and regenerate somatic embryos. To that end, the next stage of research would be the optimization of culture medium to induce somatic embryogenesis.

Acknowledgement. The authors would like to thank the University of Jambi, Indonesia, for financial support to conduct this research through the Competitive Internal Research Grant for Academic Staffs.

REFERENCES

[1] Ahmed, W., T. Feyissa and T. Disasa, (2013): Somatic embryogenesis of a coffee (*Coffea arabica* L.) hybrid using leaf explants. The Journal of Horticultural Science and Biotechnology, 88: 469-475.

[2] Ardiyani, F., (2015): Morphological characterization and identification of *Coffea liberica* callus of somatic embryogenesis propagation. Pelita Perkebunan, 31: 81-89.

[3] Bartos, P.M.C., Gomes, H.T., do Amaral, L.I.V., Teixeira, J.B., Scherwinski-Pereira, J.E., (2018): Biochemical events during somatic embryogenesis in *Coffea arabica* L. 3 Biotech, 8(4): 209.

[4] Baskaran, P. and J. Van Staden, (2017): Ultrastructure of somatic embryo development and plant propagation for *Lachenalia montana*. South African Journal of Botany, 109: 269-274.

[5] Basnayake, S. W. V., R. Moyle and R. G. Birch, (2011): Embryogenic callus proliferation and regeneration conditions for genetic transformation of diverse sugarcane cultivars. Plant Cell Reports, 30: 439-448.

[6] Campos, N. A., B. Panis and S. C. Carpentier, (2017): Somatic embryogenesis in coffee: The evolution of

biotechnology and the integration of omics technologies offer great opportunities. Frontiers in Plant Science, 8: 1460.

[7] de Rezende, J. C., E. A. Ferreira, M. Pasqual, F. Villa, C. E. Botelho and S. P. de Carvalho, (2008): Development of *Coffea arabica* L. seedling obtained from direct somatic embryogenesis. Coffee Science, 3: 30-37.

[8] Gatica-Arias, A. M., G. Arrieta-Espinoza and A. M. Espinoza Esquivel, (2008): Plant regeneration via indirect somatic embryogenesis and optimisation of genetic transformation in *Coffea arabica* L. cvs. Caturra and Catuaí. Electronic Journal of Biotechnology, 11: 1-12.

[9] Georget, F., P. Courtel, E. M. Garcia, M. Hidalgo, E. Alpizar, J.-C. Breitler, B. Bertranda and H. Etienne, (2017): Somatic embryogenesis-derived coffee plantlets can be efficiently propagated by horticultural rooted mini-cuttings: A boost for somatic embryogenesis. Scientia Horticulturae, 216: 177-185.

[10] Guo, Y. and Z. X. Zhang, (2005): Establishment and plant regenerations of somatic embryogenic cell suspension cultures of the *Zingiber officinale* Rosc. Scientia Horticulturae, 107: 90-96.

[11] Hartati, R.S., Ibrahim, M.S.D., (2017): Teknologi kultur jaringan untuk perbanyakan bahan tanaman kopi Arabika. http://new.litbang.pertanian.go.id/info-teknologi/3064/ accessed on July, 2019.

[12] Kartika, E., L. Lizawati and Z. Zulkarnain, (2019): *In vitro* callus formation from male inflorescences of oil palm (*Elaeis guineensis* Jacq.) by the application of Picloram. Analele Universităţii din Oradea, Fascicula Biologie, 26: 57-61.

[13] Kumar, V., M. M. Naidu and G. A. Ravishankar, (2006): Developments in coffee biotechnology - *in vitro* plant propagation and crop improvement. Plant Cell, Tissue and Organ Culture, 87: 49-65.

[14] Landey, R. B., A. Cenci, F. Georget, B. Bertrand, G. Camayo, E. Dechamp, J. C. Herrera, S. Santoni, P. Lashermes, J. Simpson and H. Etienne, (2013): High genetic and epigenetic stability in *Coffea arabica* plants derived from embryogenic suspensions and secondary embryogenesis as revealed by AFLP, MSAP and the phenotypic variation rate. Plos ONE, 8: e56372.

[15] Muniswamy, B., H. L. Sreenath, S. D. Samuel and J. Jayarama, (2015): *In vitro* multiplication of *Coffea arabica* F1 hybrid (S.2800) and its performance in the field. Journal of Plantation Crops, 43: 225-230.

[16] Murashige, T. and F. Skoog, (1962): A revised medium for rapid growth and bio assays with tobacco tissue cultures. Physiologia Plantarum, 15: 473-497.

[17] Padua, M. S., L. V. Paiva, L. C. Silva, K. G. Livramento, E. Alves and A. H. F. Castro, (2014): Morphological characteristics and cell viability of coffee plants calli. Ciência Rural, 44: 660-665.

[18] Rahman, M. M., M. Mahmood, N. Abdullah, N. A. Shaharuddin and W. Parvin, (2017): Somatic embryogenesis and subsequent plant regeneration from zygotic embryo derived callus of rubber (*Hevea brasiliensis* Muell. Arg.). Plant Tissue Culture and Biotechnology, 27: 51-61.

[19] Riyadi, I. and Tirtoboma, (2004): Pengaruh 2,4-D terhadap induksi embrio somatik kopi arabika. Buletin Plasma Nutfah, 10: 82-89.

[20] Romeida, A., D. W. Ganefianti and Rustikawati, (2016): Embryogenic callus induction of Pencil Orchid (*Papilionanthe hookeriana* Rchb.f.) through *in vitro* culture. International Journal on Advanced Science Engineering Information Technology, 6: 196-200.

[21] Santana-Buzzy, N., R. Rojas-Herrera, R. M. Galaz-Ávalos, J. R. Ku-Cauich, J. Mijangos-Cortés, L. C. Gutiérrez-Pacheco, A. Canto, F. Quiroz-Figueroa and V. M. Loyola-Vargas, (2007): Advances in coffee tissue culture and its practical applications. In Vitro Cellular and Developmental Biology - Plant, 43: 507-520.

[22] Silva, A. T., D. Barduche, K. G. do Livramento and L. V. Paiva, (2015): A putative BABY BOOM-like gene (CaBBM) is expressed in embryogenic calli and embryogenic cell suspension culture of *Coffea arabica* L. In Vitro Cellular and Developmental Biology - Plant, 51: 93-101.

[23] Tapingkae, T., Z. Zulkarnain, M. Kawaguchi, T. Ikeda and A. Taji, (2012): Somatic (asexual) procedures (haploids, protoplasts, cell selection) and their applications. *Dalam* A. Altman and P. M. Hasegawa [eds.], Plant Biotechnology and Agriculture: Prospects for 21st Century, 141-162. Elsevier Inc., USA.

[24] Zhong, D. Y., Y. Y. Zhu, Q. Liu, T. Zhou and D. G. Zhao, (2011): Production of embryogenic callus and plant regeneration from elite Guizhou Waxy Maize inbred lines. Agricultural Sciences in China, 10: 490-498.

[25] Zulkarnain and Lizawati, (2011): Proliferasi kalus dari eksplan hipokotil dan kotiledon tanaman Jarak Pagar (*Jatropha curcas* L.) pada pemberian 2,4-D. Jurnal Natur Indonesia, 14: 19-25.

[26] Zulkarnain, Z., (2007): Pretreatment stress enhances embryogenic callus production in anther culture of Sturt's Desert Pea (*Swainsona formosa*). HAYATI Journal of Biosciences 14: 28-30.

[27] Zulkarnain, Z., T. Tapingkae and A. Taji, (2015): Applications of *in vitro* techniques in plant breeding. *Dalam* J. M. Al-Khayri, S. M. Jain and D. V. Johnson [eds.], Advances in Plant Breeding Strategies: Breeding, Biotechnology and Molecular Tools (Volume 1). Springer International Publishing, Switzerland.

Received: 12 August 2019
Accepted: 4 March 2020
Published Online: 10 March 2020
Analele Universităţii din Oradea, Fascicula Biologie
http://www.bioresearch.ro/revistaen.html
Print-ISSN: 1224-5119
e-ISSN: 1844-7589
CD-ISSN: 1842-6433
University of Oradea Publishing House