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MICROPROPAGATION OF RARE KOPYOR DWARF COCONUT (*Cocos nucifera* L.) THROUGH SOMATIC EMBRYOGENESIS: INDUCTION AND MORPHOLOGICAL CALLUS

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Abstract- Kopyor coconut is a natural mutant that has abnormal endosperm development and cannot be propagated by conventional technique. To rescue the population of kopyor dwarf coconut in nature, micropropagation by somatic embryogenesis has been done through callus induction. Embryogenic calluses were induced from zygotic embryo explants in the media contained 5-15 mg. L^{-1} 2,4-D (2,4-dichlorophenoxyacetic acid) or 0.5-1.0 mg. L^{-1} picloram. Explants were cultured in the Eeuwens media consisted of 2.5 $g.L^{-1}$ activated carbon, 60 $g.L^{-1}$ sucrose, and 7 $g.L^{-1}$ agar. The induction of calluses was strongly influence by the presence of 2,4-D or picloram. On culture medium without 2,4-D or picloram, explants presented the development of shoot growth, but no calluses was observed. Growth regulator 2,4-D could induce embriogenic callus in the ranged concentration from 5 to 15 $mg.L^{-1}$. A high concentration of 2,4-D (above 15 $mg.L^{-1}$) caused explants necrotic and led to the death tissue. Concentration below those range resulted in a small number of callus or no response. Picloram showed less effective in producing embriogenic callus compared to 2,4-D. Morphological analyses revealed that embriogenic callus had friable and nodular structure, compact, with white yellowish colour, while non-embriogenic callus showed spongy structure and white colour.

Key words: auxin, callus multiplication, picloram, somatic embryo, 2,4-D.

INTRODUCTION

Kopyor dwarf coconut which produced a nut with endosperm is soft and incompact, found very rare in Indonesia and only exist in Java Island. Unlike normal coconut, kopyor coconut water has endosperm particles floating in it. The kopyor coconut water is regarded as a delicious and nutritious natural drink. These characters make kopyor coconut interesting to be developed further more. Kopyor coconut is an expensive delicacy and its planting material highly priced.

Indonesian farmers planted kopyor dwarf coconut by planting seedling nut from hetero-zygote coconut tree. This tree produces two kinds of nuts, normal and kopyor coconut in its bunches. By this method and depending on where they are planted, the percentage of kopyor nuts produced very low, only 1-2 fruits per bunch. When planted as a solid plantation so that cross-pollination among them is virtually assured, higher yielded is expected than there are interplanted with other varieties. Until now, the pure kopyor seedling is very expensive and it will not be reached by farmer budget. Although kopyor dwarf coconut contains an apparently normal embryo, it fails to germinate properly as the endosperm contains substances which are obviously lethal. Thus, pure-bearing kopyor coconut palms have not been obtained in nature. The only way, to germinate the embryo is through *in vitro* culture.

Propagation of kopyor coconut by embryo culture (embryo rescue) in Indonesia has been done since 1980s (Tahardi and Warga Dalem, 1982) and recently has been developed as commercial kopyor coconut seedling. However, this technique only produced one seedling from one embryo explant cultured, so an alternative *in vitro* method should be undertaken to multiply plantlet rate to increase the seedling obtained. Development of a reliable clonal propagation such as somatic embryogenesis would provide a rapid multiplication for kopyor dwarf coconut.

There are several reports on normal coconut propagation through somatic embryo which is formed from callus (Blake, 1990; Verdeil *et al.*, 1992; Samosir *et al.*, 1998). According to those published material, callus could be induced from different sources of explants such as inflorescence and leaf (Blake, 1990; Verdeil *et al.*, 1992), plumule (Hornung, 1995), and embryo (Karunaratne *et al.*, 1989; Samosir *et al.*, 1998). Alongside source of explants, callus initiation of normal coconut

requires the presence of an auxin such as 2,4-D and picloram. Even though many works on somatic embryogenesis have been done in normal coconut, no research on it in kopyor dwarf coconut has been reported. Therefore, the present work was to investigate the response of zygotic embryo dwarf coconut explants in the induction callus medium and their callus characteristic based on morphological parameters to know their ability in the formation of somatic embryos.

MATERIAL AND METHODS

Plant materials: Zygotic embryos were used as source of explants. The embryos were excised from 11-12 month-old kopyor dwarf coconut fruits, collected from Jember, East Java, Indonesia. The embryo that still enclosed with endosperm was sterilized in 1.0% sodium hypochlorite (NaOCl) for 10 min and rinsed three times with distilled sterile water. They were then excised from the endosperm and sterilized using 0.5% NaOCl for 5 min, and washed three times with distilled sterile water. They were placed in test tube containing 10 ml liquid Eeuwens medium and incubated in the culture room without light at 25 ± 2°C. The embryos that germinated, indicated by plumule or radical growth, were selected as explants.

Embyiogenic callus induction:

Effect of 2,4-D. The germinated embryo was sliced into two parts after the haustorium discarded. The embryo slices were cultured in the basal Eeuwens medium containing 2,4-D (5, 10, 15 $mg.L^{-1}$), 2.5 $g.L^{-1}$ activated carbon, 45 $g.L^{-1}$ sucrose, 7 agar $g.L^{-1}$, and amino acid and in the medium without 2,4-D as a control. The pH of medium was adjusted to 5.8 before autoclaving for 20 min at 121°C. The experiment was arranged in Completely Randomized Design with three replications and every replication consisted of ten samples. The cultures were incubated in the dark room at 25 ± 2°C and subcultured every month.

Effect of picloram. Explants of embryo slices were cultured in the Eeuwens media containing picloram (0.5 and 1 $mg.L^{-1}$), 2.5 $g.L^{-1}$ activated carbon, 45 $g.L^{-1}$ sucrose, 7 $g.L^{-1}$agar, and amino acid and in the medium without picloram. Each concentration of picloram was replicated three times in which each replication consisted of ten samples. The cultures were kept in the dark at 25 ± 2°C.

All data were analysed based on Analysis of Variance (ANOVA) which were carried out using the Statistical Analysis System (SAS) software.The differences among treatment means were detected by Duncan Multiple Range Test (DMRT) in the significant level of 5%.

Embryogenic callus proliferation. To proliferate the embryogenic callus, culture was transferred into a new Eeuwens medium with decreasing level of 2,4-D or picloram. The level of 2,4-D or picloram gradually was reduced to half its previous level starting on the second transfer. Transfers were carried out every four weeks. Embryogenic callus was maintained in darkness at 25 ± 2°C for 2-3 months before transferred to the induction medium of somatic embryogenesis.

Somatic embryo formation. Nodular calluses from callus induction media containing 2,4-D were transferred into media with low level of 2,4-D (2.5 $mg.L^{-1}$) and supplemented with BAP 10 $mg.L^{-1}$, 60 $g.L^{-1}$ sucrose, 2.5 $g.L^{-1}$ activated carbon, 7 $g.L^{-1}$ agar to induce somatic embryogenesis. Meanwhile, embryogenic calluses from media containing picloram were transferred to the hormone-free media. Cultures were transferred to fresh media every four weeks. They were incubated at 25 ± 2°C in a light room from cool white florescent lamps with 8 hr photoperiod for further development.

Morphological analysis. Callus was examined morphologically using a digital microscope NTSC System- DC2-456 type which is connected to the computer. The callus was observed at every phase of its development. Observation was further done on early formation of somatic embryo in the globular phase.

RESULTS

Embryogenic callus and somatic embryo formation: Callus of kopyor dwarf coconut could be induced from explants of zygotic embryo slices in the Eeuwens medium containing auxin (2,4-D or picloram). Explants started enlarged (Figure 1a) after 1-2 weeks of culture and formed callus (Figure 1b) at 4-5 weeks of culture, in which that it will be cited as initial callus. The callus continued growing and showed active cell division and proliferation leading to the nodular callus formation. About 8-10 weeks, the nodular calluses became globular structures and from these the globular somatic embryos were formed.

The presence of growth regulator (auxin) such as 2,4-D or picloram was required to induce embryogenic callus of kopyor dwarf coconut. In the media without 2,4-D or picloram, explants did not show any obvious growth to form callus (Table 1). The explants were just swollen but no further development. The concentration of 2,4-D in the range of 5-

15 mg.L^{-1} or picloram of 0.5-1.0 mg.L^{-1} could induce callus formation. However, different 2,4-D or picloram concentrations did not have a clear effect on the formation of that callus (Table 1). Even though picloram could be used to induce kopyor dwarf coconut callus, the callus percentage was lower compare to 2,4-D and tended to browning. The 2,4-D did not affect the diameter callus but significantly affected the fresh weight of callus while picloram did not have effect on both diameter and fresh weight.

The initial medium that containing different concentrations of 2,4-D showed different effect on the production of globular embryos. Embryogenic callus originated from medium that supplemented with 10 mg. L^{-1} 2,4-D was found to be better than 5 mg.L^{-1} or 15 mg.L^{-1} in the production of somatic embryos in the globular phase (Figure 2). For picloram, all calluses were transferred to the hormone- free media because of the quality was not good enough. Embryogenic callus from picloram concentration of 0.5 mg.L^{-1} produced somatic embryos was higher than 1.0 mg.L^{-1}. Between the two, 2,4-D apparently was better in the somatic embryo formation.

Morphology of callus. Callus initiation of kopyor dwarf coconut was occurred on the surface of explants. On an average, 80-90% per cent of zygotic embryos explants gave calluses in media containing 2,4-D. Observation showed that there were two types of calluses. First, callus had a loose consistency, a white colour, smooth appearance (Figure 3c). The callus continued proliferate to form a spongy structure (Figure 3b) without any further development that leading to the induction of somatic embryogenesis. The type of callus that did not have embryogenic or morphogenic capability, we referred to these as non-embryogenic callus. Second, callus had a hard consistency, cream coloured or white yellowish colour, friable, and nodular structure (Figure 3e). The callus showed active cell proliferation leading to the development of globular or elongated structures. This callus had embryogenic capability and we mentioned it as embryogenic callus. All somatic embryos formation were occurred from that embryogenic calluses (Figure 3 f).

DISCUSSION

Formation of kopyor dwarf coconut callus from zygotic embryo explants started at 4-5 weeks of culture. In the normal coconut, the same source of explants with those in kopyor dwarf coconut expanded and produced callus during the first 4 weeks of culture (Samosir *et al.*, 1998). Karunaratne and Periyapperuma (1989) noticed that callus formation of coconut var. Typical take placed within 2-4 weeks of culture. It appeared that formation of most coconut callus occurred within 4 weeks in culture, as observed by Saenz *et al.* (2006) in the plumule explants which showed partial dedifferentiation and meristematic cell proliferation leading to the development of callus with a translucent appearance at day 45.

Figure 1. Callus formation of embryo explants of kopyor dwarf coconut in the Eeuwens medium with 2,4-D. a. Embryo explants started swollen and formed callus, b. Embryogenic callus in the 10 mg.l^{-1} 2,4-D media, c. Embryogenic callus that developed to form somatic embryo in the globular phase.

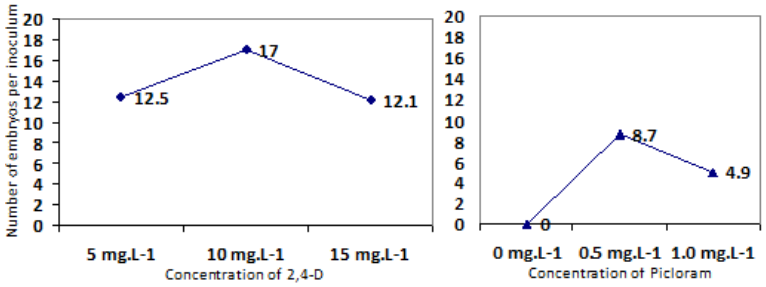

Figure 2. Number of embryos of kopyor dwarf coconut per inoculum in the globular phase derived from embryogenic callus in the Eeuwens media containing 2,4-D and picloram.

Table 1. Induction and growth of kopyor dwarf coconut callus in the Eeuwens solid media containing 2,4-D and picloram.

Auxin	% Embriogenic callus	Callus diameter (cm)	Fresh weight of callus (g)
2,4-D:			
0.0	0.00	0.00	0.000
5 mg/l	98.00	1.30	0.7675b*
10 mg/l	88.87	1.27	0.8205b
15 mg/l	91.67	0.90	0.5485a
Picloram:			
0.0	0.00	0.00	0.000
0.5 mg/l	58.35	1.13	0.1340
1.0 mg/l	61.13	1.15	0.3705

*Means followed by the same letter in the same column and treatment are not significantly different at the 5% Duncan Test

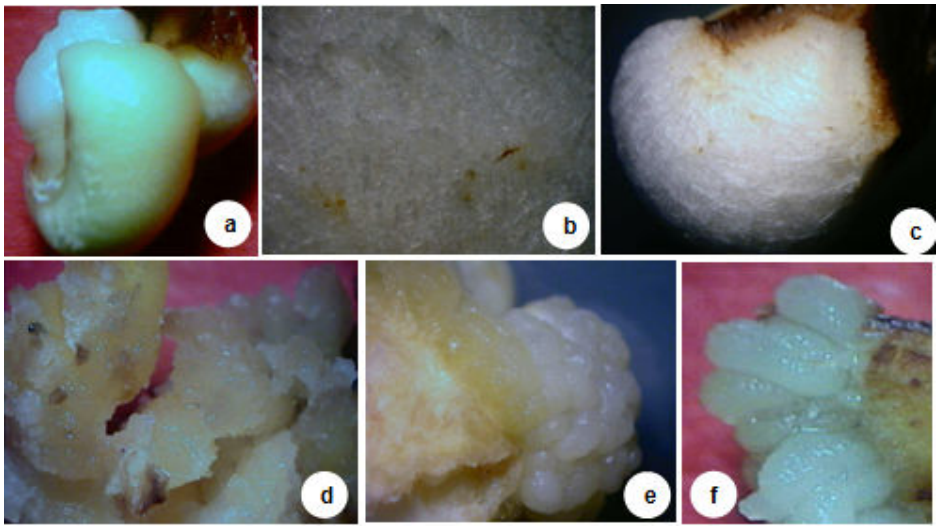

Figure 3. Morphology of embriogenic and non-embryogenic callus of kopyor dwarf coconut, a. Explant of zygotic embryo that excised from nut of kopyor dwarf coconut, b. A spongy structure of non-embryogenic callus, c. Non-embryogenic callus that grew bigger without cell differentiation, d. Creamy and compact embryogenic callus, e. Nodular structures that emerged from surface of callus explants, f. Globular somatic embryos derived from nodular embryogenic calluses.

Most of zygotic embryo explants enlarged and formed calluses that originated from tissue in the surrounding area of the meristem apical (Figure 1a-b) which was described as shoot and root apices by Branton and Blake (1983) or as cotyledonary node by Karunaratne and Periyapperuma (1989). Initially, the calluses appeared watery (Figure 1b) but overtime with more subcultures the form of the calluses became compact, friable and nodular. The calluses which maintained in a media similar to that used for initiation grew bigger and proliferated. Once nodular calluses were transferred to the embryogenesis induction media formed globular embryos, and this embryos clearly separated and let to develope individually.

Induction of kopyor dwarf coconut callus required an auxin plant growth regulator such as 2,4-D or picloram. The 2,4-D was better used than picloram and the best concentration of 2,4-D was 5 $mg.L^{-1}$. Usually callus formation of normal coconut is observed in the presence of a high level of 2,4-D which results in the development of proembryoids (Blake, 1990). However, high level of 2,4-D caused the explants of kopyor dwarf coconut necrotic. Even though 2,4-D is required to stimulate callus formation, excessive levels tend to induce browning and inhibit growth of cultures (Ebert and Taylor, 1990). The present results suggested that concentration of 2,4-D for callus formation was 5 to 15 $mg.L^{-1}$. In the concentration less than 5 $mg.L^{-1}$, no response was observed. Clearly that zygotic embryo explants of dwarf coconut need a certain

amount of 2,4-D to form calluses. Several monocot plant species showed the same response to the 2,4-D but with different range of concentration. Liu *et al.* (2006) reported that no optimal 2,4-D concentration could be determined for the induction of perennial ryegrass calluses under a range of 5-12 mg. L^{-1}, however, different response occurred at the concentration 2 and 12 mg. L^{-1}. In wheat, at the low concentration of 2,4-D explants showed swelling only. Callus produced in the media containing 2,4-D at the range of 1.0-4.0 mg.L^{-1}, above these concentration resulted non-embryogenic calluses (Yasmin *et al.*, 2001).

Quality of callus is believed to be related to the embryogenic or morphogenic capability. In this study, we found two types of calluses that were produced by either medium containing 2,4-D or picloram which show different characteristics. First, embryogenic calluses are characterized by friable, hard, and nodular structures that easily discernible with the surrounding tissue on the surface of explants. In some monocot plants the embryogenic callus was characterized by its appearance such as white-yellowish, compact, nodular, and friable (Gill *et al.*, 2004; Amna Noor *et al.*, 2005; Sumaryono *et al.*, 2008). Occurrence of embryogenic calluses are well described in normal coconut (Saenz *et al.*, 2006). They are compact structures, hard, white in colour, which develop from the meristematic cells and start grouping into meristematic nodules. Later on, Saenz *et al.* (2009) described them as compact and smooth small structures that appeared on the surface of the translucent structures. Initially the structures were globular and then became elongated and from which somatic embryos were formed. This type of calluses has a high capability to form somatic embryos (Zulkarnaim, 2004). Once embryogenic calluses transferred to the embryo somatic induction media formed embryoids and started to develop individually.

The other type is non-embryogenic callus which has characteristic such as white colour, spongy, soft, and unorganized structure. We used non-embryogenic term to describe callus that doesn't have any embryogenic or organogenic ability and discarded during subculture. Other authors may be used non-embryogenic with a slightly different meaning. Liu *et al*. (1992) described non-embryogenic callus in sweet potato (*Ipomea triloba* L.) as organogenic callus which has a capability to form shoot via organogenesis process. Meanwhile, Yasmin *et al.* (2001) reported that only embryogenic callus produced embryoids while non-embryogenic callus failed to form embryoid, this callus lost their proliferative capability and turned brown (Zulkarnaim, 2004).

It has been known that growth regulator play a major role in the subsequent development of callus into somatic embryo. The induction of somatic embryogenesis is generally carried out by reduction of auxin concentration and addition of cytokinins in the culture medium. Although auxin is required for proliferation of embryogenic callus but is inhibitory for the development of this callus into somatic embryo (Nomura and Komamine, 1995; Filonova *et al.*, 2000). However, in normal coconut the formation of proembryos occurred when callus was still cultured in the medium containing high levels of 2,4-D (Verdeil *et al.*, 1994). In this study, some globular embryos have been formed in the proliferation media in the second subculture. Others were formed upon the transfer of the embryogenic callus to an embryo somatic induction medium with low level of 2,4-D. The phenomenon of globular embryo formation when the callus was still in the callus induction was also occurred in the date palm (Eke *et al.*, 2005). The differentiation callus into globular embryo that occurred during in the callus induction while others in the somatic induction or in the hormone free medium, however, cannot be well understood. It may be embryogenic callus usually consist of a mixture of single cells and cell aggregates with unsynchronised development. We noticed that the globular embryos which were formed too early whereas the medium still contained a high level of 2,4-D tended to develop into root. Von Arnold *et al.* (2002) described that transition from callus (PEMs) to embryos related with appropriate developmental stage of the culture. The embryos should not be transferred to maturation media before they have reached that stage. Prematuration of somatic embryos cause inability of embryogenic cell to form well-developed somatic embryos.

This work demonstrates that induction of embryogenic callus of kopyor dwarf coconut and formation of somatic embryos on the globular phase could be controlled by level of auxin concentration. The embryogenic callus can be identified by its morphology and distinguished from the non-embryogenic one.

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