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The Effect of Different Levels and Sources of Auxin and Cytokinin to Callus Formation on Soybean Anther Culture

Pengaruh Berbagai Takaran dan Sumber Auksin dan Sitokinin terhadap Pembentukan Kalus pada Kultur Antera Kedelai

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ABSTRACT

This investigation was aimed at studying the effect of types and concentrations of auxins and cytokinins on the growth and development of anthers of two soybean cultivars, Merubetiri and Wilis cultured *in vitro*. The trial was conducted at the Plant Biotechnology Laboratory, Agricultural Faculty, University of Jambi. Anthers were cultured on MS solid medium provided with IAA, 2,4-D or NAA as auxin source in combination with BAP or kinetin as cytokinin source. Each growth regulators was tested at 0, 5, 10, 15 and 20 µM. The experiment was placed in a completely randomized design with five replicates. Each replicates consisted of 8 to 10 anthers obtained from the same floral bud. Cultures were placed in a light intensity of 50 µmol $m^{-2}.s^{-1}$ and 16-hour photoperiod at 25±1 ^{o}C. Observation was done weekly for 8 weeks of culture. Results indicated that response showed by anthers cultured on medium supplemented with 2,4-D+BAP, IAA+BAP and NAA+BAP, in the form of callus proliferation, occurred within 5-18 days of culture initiation. Meanwhile, with 2,4-D+kinetin, IAA+kinetin and NAA+kinetin, callus proliferation took place within 4-16 days of culture initiation. Callus formation was preceded by a swollen on the surface of anthers, followed by changing in color from light green to brownish. Following this, anther wall turned into amorphous shape, before it was finally covered by a white, cream or light green callus mass. Initially, the callus showed friable or compact structure, but following two weeks of proliferation all callus showed compact structure. Among growth regulators tested, combination involving 2,4-D produced more callus than other combinations. In addition, of the two cultivars tested, Merubetiri showed better response compared to Wilis.

Keywords: *growth regulators, anther culture, in vitro culture, soybean, Glycine max*

ABSTRAK

Penelitian ini bertujuan untuk mengetahui pengaruh berbagai jenis dan konsentrasi zat pengatur tumbuh auksin dan sitokinin terhadap pertumbuhan dan perkembangan antera dua kultivar kedelai, Merubetiri dan Wilis, pada kultur in vitro. Penelitian dilaksanakan di Laboratorium Bioteknologi Tanaman, Fakultas Pertanian Universitas Jambi. Antera yang dari dua kultivar kedelai yang diuji dikulturkan pada medium MS padat yang dilengkapi dengan IAA, 2,4-D atau NAA sebagai sumber auksin yang dikombinasikan dengan BAP atau kinetin sebagai sumber sitokinin. Masing-masing zat pengatur tumbuh diberikan pada konsentrasi 0, 5, 10, 15 and 20 µM. Percobaan ini menggunakan rancangan acak lengkap dengan lima ulangan. Setiap ulangan terdiri atas 8-10 antera yang berasal dari kuncup bunga yang sama. Kultur dipelihara di dalam ruangan dengan intensitas cahaya lebih-kurang 50 µmol $m^{2}.s^{-1}$ dan fotoperiodesitas 16 jam per hari pada suhu 25± 1 ^{o}C. Pengamatan dilakukan setiap minggu selama 8 minggu. Hasil penelitian menunjukkan bahwa respons yang diperlihatkan oleh antera yang dikulturkan pada medium yang dilengkapi dengan 2,4-D+BAP, IAA+BAP dan NAA+BAP adalah berupa proliferasi kalus, yang berlangsung dalam waktu 4-16 minggu setelah inisiasi kultur. Pembentukan kalus didahului oleh timbulnya pembengkakan pada permukaan antera, diikuti oleh perubahan warna dari hijau muda menjadi kecoklatan. Selanjutnya, dinding antera berubah bentuk menjadi tidak beraturan, sebelum akhirnya diselimuti oleh massa kalus berwarna putih, krem atau hijau muda. Pada awal perkembangannya, kalus memperlihatkan struktur yang remah dan sebagian kompak, namun setelah dua minggu berproliferasi seluruh kalus memperlihatkan struktur yang kompak. Di antara zat pengatur tumbuh yang diuji, kombinasi yang melibatkan 2,4-D menghasilkan kalus yang lebih banyak dibandingkan kombinasi yang lain. Di samping itu, dari dua kultivar yang diuji, Merubetiri memperlihatkan respons yang lebih baik dibandingkan Wilis.

Kata kunci: *zat pengatur tumbuh, kultur antera, kultur in vitro, kedelai, Glycine maX*

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INTRODUCTION

Haploid technology offers more advantages than conventional method. By this technique, homozigous plants could be developed within one generation, while conventional method requires selection processes involving 5-6 generations to produce homozigous plants (Taji *et al.*, 2002). Haploid individuals also provide an excellent example when studying induced mutagenesis, where recessive traits can be easily detected (Seguí-Simarro and Nuez, 2008b). A number of recessive traits such as tolerance to unfavourable conditions, such as drought, cold, heavy metals or low nutrients, are amongst recessive traits that can be detected promptly in haploid plants (Tapingkae *et al.*, 2012). Further, problems associated with outcrossing and self incompatibility can also be overcome by the use of haploid technology.

Haploid plants can be obtained through anther culture. Anthers obtained from young floral buds may be aseptically isolated and used as plant materials in tissue culture. Immature microspores within these anthers may be induced to grow and give rise to complete plants under favourable conditions. Since microspores are haploid, plants regenerated from microspore cells will also be haploid. Haploid plants have no homologous set with which to pair; the normal pairing of chromosomes during meiosis cannot take place. Consequently, sterile plants or plants with non-functional male sexual organs are produced. When the chromosome complements are artificially doubled, e.g. using antimitotic chemicals such as colchisin or oryzalin, the regenerated plants will be doubled-haploid. As with homozigous haploids, the regenerated doubled-haploid individuals are also homozigous. The difference is that the doubled-haploid plants are fertile, and therefore can be propagated sexually.

Haploid plants regenerated from tissue system culture were first reported by Guha and Maheshwari (1964) on *Datura inoxia* and followed by Nitsch and Nitsch (1969) on tobacco (*Nicotiana tabacum*). Since then, success has also been reported on various crops such as *Lupinus* spp. (Bayliss *et al.*, 2004), *Glycine max*, *Vigna unguiculata*, *Psophocarpus tetragonolobus*, *Albizzia lebbeck* and *Peltophorum pterocarpum* (Crosser *et al.*, 2006), *Brassica* sp. (Alam *et al.*, 2009), *Oryza sativa* (Khatun *et al*., 2012) and *Populus* x *beijingensis* (Li *et al.*, 2013).

The application of plant growth regulators to culture medium is necessary for the successful induction of microspore embryogenesis. Auxin and cytokinin are the two most extensively used growth regulators in the anther culture of a wide range of plant species. Auxin such as 2,4-dichlorophenoxyacetic acid (2,4-D) was usually applied in the anther culture of *Triticum aestivum* (El-Hennawy *et al.*, 2011) and *Brassica napus* (Ardebili *et al.*, 2011). In addition, vigorous green plantlets were regenerated from microspore culture of *Hordeum vulgare* in the presence of auxins such as indoleacetic acid (IAA) or naphthaleneacetic acid (NAA) (Castillo *et al.*, 2000).

Kinetin and benzylamino purine (BAP) are two cytokinins that promote shoot regeneration from within callus derived from *Oryza sativa* (Bishnoi *et al.*, 2000) and embryogenesis in *Cucumis sativus* L. (Hamidvand *et al.*, 2013) anther cultures. In addition, Rukmini *et al.* (2013) claimed that the combination of kinetin+BAP+NAA proved to be optimal for callus induction and green plant regeneration in two Indica rice (*Oryza sativa* L.) hybrids - Ajay and Rajalaxmi.

This study was aimed at investigating the effect of different levels and sources of auxins and cytokinins on the growth and development of soybean cv. Merubetiri and Wilis anthers cultured *in vitro*.

METHODOLOGY

Stock plant preparation

The stock plants used in this investigation were two soybean cultivars, i.e. Merubetiri and Wilis grown in glasshouse. Seeds were germinated and grown in black polyethylene plastic bags. Care of plants followed general cultivation technique including watering, pests and diseases controlling, weeding and fertilizer application to obtain healthy growth. Seed germination were carried out every 3-4 weeks to ensure adequate availability of floral buds during investigation.

Explant source

Anthers obtained from 2.5-3.5 mm long floral buds were used as planting materials in this study. Floral buds from glasshouse-grown plants were isolated and dipped in 70% alcohol for 10 seconds. Following from this, sepal and petal were carefully removed and anthers were separated from filaments prior to culture in prepared media.

Culture medium

In this study we used solid MS (Murashige and Skoog, 1962) basal medium supplemented with vitamins and 3% sucrose, and the pH of the medium was adjusted to 5.6 ± 0.2. Prior to sterilizaton in autoclave at 1.1 kg cm^{-1} (103 kPa) and temperature of 121 ^{o}C for 20 minutes, 8 g Bacto Bitek™ agar was added to the medium.

Variables tested

Growth regulators tested were IAA, NAA or 2,4-D (each at 0, 5, 10, 15 and 20 μM) as auxin sources, in combination with BAP or kinetin (each at 0, 5, 10, 15 and 20 μM) as cytokinin sources. Therefore, there were 25 treatment combinations, each repeated 4 times resulted in 100 experimental units in a Simple Completely Randomized Design. Each experimental unit consisted of 4 culture flasks containing one immature leaf segment explants each.

Culture maintenance and observation

Cultured anthers were kept in a growth room with temperature of 25±1^{o}C. Photoperiod was adjusted to 16

hours per day and light intensity approximately 50 μmol $m^{-2}.s^{-1}$ obtained from fluorescent lamp.

Anthers growth and development were observed daily for eight weeks of culture period. Variables observed were the percentage of explants forming callus, time to callus formation following culture initiation, and the characteristics of callus (colour and structure).

Quantitative data were analyzed by ranking of mean method and presented in the form of tables, whereas qualitatif data were presented visually in the form of picture/ photograph.

RESULTS AND DISCUSSION

Anthers from the two soybean cultivars (Merubetiri and Willis) cultured on solid MS medium supplemented with IAA, NAA or 2,4-D in combination with BAP or kinetin showed callus formation within 5-18 days after culture. Callus proliferation was started by swollen on the anthers surface followed by changes in their colour from light green into brownish. Following from this, the anther wall showed amorphous structure, and finally the whole anthers were covered by callus mass. Meanwhile, on non callus forming explants the colour of anthers turning into white or brown and show no further development.

The percentage of explant forming callus

Medium supplemented with IAA + BAP

On Wilis, of all tested combination, callus formation was only found on anther cultured on medium supplemented with 15μM IAA+10 μM BAP. Whereas other combinations of plant growth regulators did not show any effect on explant development. All cultured explants turning white and /or brown indicating that they are all dead without showing any callus proliferation.

The same condition was also observed on Merubetiri, in which callus formation occurred only on explants cultured on medium supplemented with 15μM IAA+10 μM BAP, 20 μM IAA+5 μM BAP and 20μM IAA+15 μM BAP. Similar as Wilis, non callus forming explants turned into white or brown and finally died.

Medium supplemented with IAA+kinetin

Similar to IAA+BAP treatment, neither anthers isolated from Wilis nor Merubetiri cultured on medium supplemented with IAA+kinetin showed satisfatory responses. On Wilis, callus was only formed on medium supplemented with 20 μM IAA+10 μM kinetin and media with 20 μM IAA+20 μM kinetin. Similar response was also shown by Merubetiri. Callus proliferation only occurred on anthers cultured on media supplemented with 5 μM IAA+15 μM kinetin and 10 μM IAA+20 μM kinetin. Meanwhile, anthers cultured on other media turned white or brown without showing any callus proliferation.

Medium supplemented with 2,4-D+BAP

On Wilis, callus formation occurred on explants cultured on medium supplemented with 5 μM 2,4-D+15 μM BAP, 10 μM 2,4-D without BAP dan 20 μM 2,4-D without BAP. Meanwhile, on Merubetiri, callus were observed on anthers cultured on medium with 20 μM BAP without 2,4-D, 5 μM 2,4-D+10 μM BAP, 15 μM 2,4-D+10 μM BAP, 15 μM 2,4-D+15 μM BAP, 20 μM 2,4-D+without BAP and 20 μM 2,4-D+10 μM BAP (Table 1). Explants cultured on other media did not show any response, but their colour turned white and/or brownish and died eventualy.

Medium supplemented with 2,4-D + kinetin

The presence of 2,4-D in culture medium was found to be important for callus formation on soybean anthers. This was indicated by the proliferation of callus mass on nearly all treatments except kinetin without 2,4-D, 5 μM 2,4-D without kinetin 5 μM 2,4-D+15 μM kinetin, 5 μM 2,4-D+20 μM kinetin, 10 μM 2,4-D without kinetin, 15 μM 2,4-D without kinetin, 15 μM 2,4-D+15 μM kinetin, and 15 μM 2,4-D+20 μM kinetin. Meanwhile on Merubetiri, callus proliferation occured on anthers cultured on a number of 2,4-D+kinetin combination, but medium without 2,4-D, 5 μM 2,4-D without kinetin, 15 μM 2,4-D+5 μM kinetin, 15 μM 2,4-D+20 μM kinetin, and 20 μM 2,4-D+5 μM kinetin.

Data on callus formation from anthers of the two soybean cultivars cultured on media supplemented with 2,4-D+kinetin are presented in Table 2.

Table 1. The percentage of soybean anthers forming callus when cultured on medium supplemented with 2,4-D + BAP

Plant growth regulators		Explants forming callus (%)	
2,4-D (μM)	BAP (μM)	Cv. Wilis	Cv. Merubetiri
0	20	-	20
5	10	-	20
5	15	20	-
10	0	20	-
15	10	-	80
15	15	-	20
20	0	40	80
20	10	-	80

Table 2. The percentage of Wilis and Merubetiri anthers forming callus following culture on medium supplemented with 2,4-D+kinetin.

Plant growth regulators		Explant forming callus (%)	
2,4-D (µM)	kinetin (µM)	Cv. Wilis	Cv. Merubetiri
5	5	20	40
5	10	-	40
5	15	-	20
5	20	20	60
10	0	-	20
10	5	80	40
10	10	80	40
10	15	40	40
10	20	20	80
15	0	-	40
15	5	40	-
15	10	-	40
15	15	60	60
15	20	-	-
20	0	80	20
20	5	20	-
20	10	60	100
20	15	40	100
20	20	60	80

Medium supplemented with NAA+BAP

Anthers isolated from Wilis showed response when cultured on media supplemented with 10 µM BAP alone, 10 µM NAA+5 µM BAP, 15 µM NAA+15 µM BAP, 20 µM NAA alone, and 20 µM NAA+20 µM BAP. On the other hand, anthers obtained from Merubetiri resulted in response when exposed to culture media supplemented with 10 µM BAP without NAA, BAP 20 µM without NAA, 5 µM NAA without BAP, 5 µM NAA+10 µM BAP, 10 µM NAA without BAP, 10 µM NAA+10 µM BAP, 10 µM NAA+15 µM BAP, 10 µM NAA+20 µM BAP, 15 µM NAA+15 µM BAP, 15 µM NAA+20 µM BAP, 20 µM NAA without BAP, 20 µM NAA+5 µM BAP, and 20 µM NAA+20 µM BAP.

Data presented on Table 3 shows the percentage of anthers from the two soybean cultivars showing response when cultured on media supplemented with NAA+BAP.

Table 3. The percentage of Wilis and Merubetiri anthers forming callus following culture on medium supplemented with NAA+BAP

Plant growth regulators		Explant forming callus (%)	
NAA (µM)	BAP (µM)	Cv. Wilis	Cv. Merubetiri
0	0	-	80
0	10	20	60
0	20	-	40
5	0	-	60
5	10	-	20
10	0	-	20
10	5	40	-
10	10	-	20
10	15	-	60
10	20	-	80
15	15	20	20
15	20	-	40
20	0	20	20
20	5	-	40
20	20	20	80

Medium supplemented with NAA+kinetin

When cultured on medium supplemented with NAA+kinetin, callus proliferation was found on Wilis anther exposed to 10 µM NAA+5 µM BAP, 15 µM NAA alone, 15 µM NAA+5 µM BAP, 20 µM NAA+5 µM BAP, and 20 µM NAA+10 µM BAP. Meanwhile, on Merubetiri response was shown by anthers cultured on medium with 5 µM NAA+10 µM BAP, 5 µM NAA+20 µM BAP, 10 µM NAA+5 µM BAP, 10 µM NAA+10 µM BAP, 10 µM NAA+15 µM BAP, 10 µM NAA+20 µM BAP, 15 µM NAA without BAP, 15 µM NAA+15 µM BAP, 15 µM NAA+20 µM BAP, 20 µM NAA+5 µM BAP, and 20 µM NAA+10 µM BAP.

Table 4 shows the percentage of anthers forming callus from the two soybean cultivars when cultured on media supplemented with NAA+kinetin.

Table 4. The percentage of Wilis and Merubetiri anthers forming callus following culture on medium supplemented with NAA+kinetin.

Plant growth regulators		Explant forming callus (%)	
NAA (µM)	BAP (µM)	Cv. Wilis	Cv. Merubetiri
5	10	-	20
5	20	-	20
10	5	20	20
10	10	-	40
10	15	-	20
10	20	-	20
15	0	20	20
15	5	20	-
15	15	-	20
15	20	-	40
20	5	20	20
20	10	80	20

Table 5. Time to callus initiation on anthers of soybean cv. Wilis and cv. Merubetiri cultured on medium supplemented with BAP plus different sources of auxin.

Plant growth regulators		Time to callus initiation (days)	
Auxin	Cytokinin	Cv. Wilis	Cv. Merubetiri
0 µM 2,4-D	20 µM BAP	-	7
0 µM NAA	0 µM BAP	-	5
0 µM NAA	10 µM BAP	5	5
0 µM NAA	20 µM BAP	-	5
5 µM 2,4-D	10 µM BAP	-	7
5 µM 2,4-D	15 µM BAP	7	-
5 µM NAA	0 µM BAP	-	5
5 µM NAA	10 µM BAP	-	5
10 µM 2,4-D	0 µM BAP	7	-
10 µM NAA	0 µM BAP	-	5
10 µM NAA	5 µM BAP	7	-
10 µM NAA	10 µM BAP	-	5
10 µM NAA	15 µM BAP	-	5
10 µM NAA	20 µM BAP	-	9 - 11
15 µM 2,4-D	10 µM BAP	-	8
15 µM 2,4-D	15 µM BAP	-	7
15 µM IAA	10 µM BAP	5	14
15 µM NAA	15 µM BAP	7	5
15 µM NAA	20 µM BAP	-	5 - 11
20 µM 2,4-D	0 µM BAP	6	5 - 7
20 µM 2,4-D	10 µM BAP	-	7 - 8
20 µM IAA	5 µM BAP	-	18
20 µM IAA	15 µM BAP	-	16
20 µM NAA	0 µM BAP	5	18
20 µM NAA	5 µM BAP	-	5
20 µM NAA	20 µM BAP	-	5 - 12

The time to callus proliferation after culture initiation

Data collected during the investigation showed that callus proliferation on the surface of anthers cultured on medium supplemented with BAP occurred within 5 to 18 days after culture initiation (Table 5). Meanwhile, on medium supplemented with kinetin callus proliferation was found within 4 to 16 days following culture initiation (Table 6).

The colour of callus

In general, medium supplemented with BAP+IAA or BAP+NAA produced callus that was initially greeny transparent in colour. On the other hand, white callus was initiated on anthers cultured on medium enriched with BAP+2,4-D which then turned creamy transparent (Figure 1). On medium with IAA as auxin source, the greeny transparent callus gradually turned light green before eventually turning dark green (Figure 2A,B). Meanwhile, on medium supplemented with NAA the initially greeny transparent callus turned whitish green or light green following 2 weeks of proliferation (Figure 3).

In Wilis, the changes in colour was mostly on callus proliferated from anthers cultured on medium with NAA, whereas in Merubetiri the changes in colour was found only on callus formed on anthers cultured on medium with 20 µM NAA+20 µM BAP. In addition to whitish green, some callus regenerated on Merubetiri anthers also turned into dark green, especially those cultured on medium with 20 µM BAP alone, 15 µM NAA+20 µM BAP and 20 µM NAA+20 µM BAP, or light green on medium with 15 µM NAA+20 µM BAP, 10 µM NAA+20 µM BAP and 20 µM NAA+20 µM BAP.

Table 6. Time to callus initiation on anthers of soybean cv. Wilis and cv. Merubetiri cultured on medium supplemented with kinetin plus different sources of auxin.

Plant growth regulators		Time to callus initiation (days)	
Auxin	Cytokinin	Cv. Wilis	Cv. Merubetiri
5 µM 2,4-D	5 µM kinetin	13	9 – 10
5 µM 2,4-D	10 µM kinetin	–	10 – 12
5 µM 2,4-D	15 µM kinetin	–	9 – 10
5 µM 2,4-D	20 µM kinetin	11	6 – 10
5 µM IAA	15 µM kinetin	–	7 – 8
10 µM IAA	20 µM kinetin	–	11
5 µM NAA	10 µM kinetin	–	7
5 µM NAA	20 µM kinetin	–	6
10 µM 2,4-D	0 µM kinetin	–	9
10 µM 2,4-D	5 µM kinetin	9 – 15	11
10 µM 2,4-D	10 µM kinetin	11 – 13	12 – 14
10 µM 2,4-D	15 µM kinetin	6 – 11	9 – 10
10 µM 2,4-D	20 µM kinetin	13	5 – 9
10 µM NAA	5 µM kinetin	10	8
10 µM NAA	10 µM kinetin	–	8
10 µM NAA	15 µM kinetin	–	7
10 µM NAA	20 µM kinetin	–	7 – 8
15 µM 2,4-D	0 µM kinetin	9 – 13	8 - 10
15 µM 2,4-D	5 µM kinetin	9 – 13	
15 µM 2,4-D	10 µM kinetin	–	9 – 14
15 µM 2,4-D	15 µM kinetin	9 – 15	9 – 14
15 µM NAA	0 µM kinetin	10	6 – 7
15 µM NAA	10 µM kinetin	9 – 11	–
15 µM NAA	5 µM kinetin	6 – 9	–
15 µM NAA	15 µM kinetin	–	8
15 µM NAA	20 µM kinetin		7 – 8
20 µM 2,4-D	0 µM kinetin	7 – 9	8 – 13
20 µM 2,4-D	10 µM kinetin	6 – 16	6 – 13
20 µM 2,4-D	5 µM kinetin	12 – 14	–
20 µM 2,4-D	15 µM kinetin	7 – 10	8 – 10
20 µM 2,4-D	20 µM kinetin	4 – 10	9 – 13
20 µM IAA	15 µM kinetin	7	–
20 µM IAA	20 µM kinetin	10	–
20 µM NAA	5 µM kinetin	–	4
20 µM NAA	20 µM kinetin	–	6

Figure 1. White callus proliferated on anthers cultured on medium supplemented with 2,4-D + BAP.

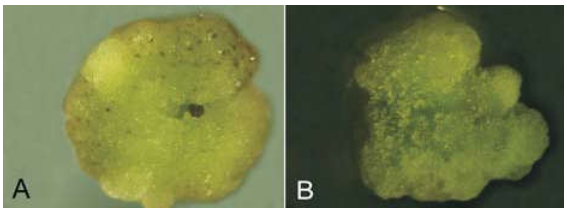

Figure 2. Light green (A) and dark green (B) callus proliferated on anthers cultured on medium supplemented with IAA + BAP.

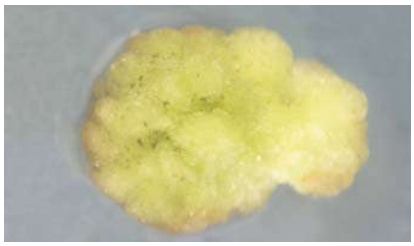

Figure 3. Whitish green callus proliferated on anthers cultured on medium supplemented with NAA + BAP.

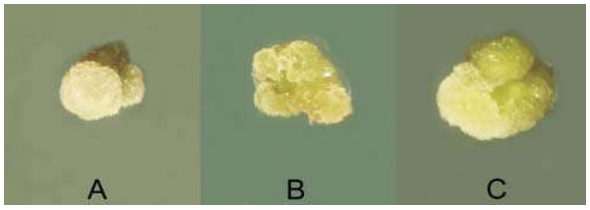

Figure 4. White, creamy yellow and greenish callus proliferated on anthers cultured on medium supplemented with kinetin as cytokinin source in combination with 2,4-D (A), IAA (B) and NAA (C) as auxin sources (2 weeks after culture initiation).

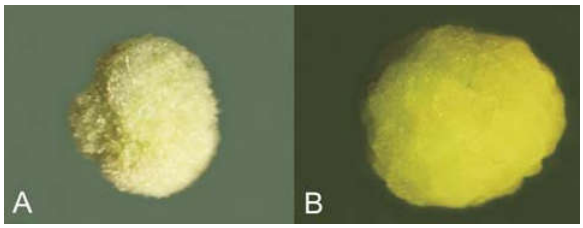

Figure 5. The compact structure of callus proliferated on anhers of soybean cv. Merubetiri cultured on medium with BAP (A) and kinetin (B) as cytokinin source (4 weeks after proliferation).

Four weeks after culture initiation, the light green callus started browning on the side exposed to culture medium. On the other hand, white to creamy yellow callus took longer time to brown, approximately 10 weeks after initiation. Meanwhile, callus proliferated from anthers of the two soybean cultivars cultured on medium supplemented with kinetin in combination with 2,4-D, IAA or NAA relatively similar in colour. In general, in spite of auxin sources, the colour of callus regenerated on medium with kinetin as cytokinin source was white transparent to creamy yellow (Figure 4).

Callus structure

At the begining of its proliferation, all callus regenerated from anthers cultured on all auxin and cytokinin combinations showed the same structure, ie compact to friable. Following 4 weeks of proliferation, however, all those callus turned into compact structure (Figure 5).

Discussion

All regenerated callus proliferated from within anthers following the broken of anther wall. This indicates that there was possibility that those callus originated from microspores within the anthers. Therefore, the ploidy level of regenerated callus presumably the same as the ploidy level of the microspores, ie haploid. This assumption, however, need to be proven by examining and counting the number of the chromosome under microscope. Another way to prove the callus was haploid was to induce embryogenesis from within callus mass. It was hoped that embryoids raised from callus were haploid and grew into complete haploid plants.

This investigation revealed that the involvement of plant growth regulators, especially auxin, in culture medium significantly affected callus formation. Among tested auxins, 2,4-D was more effective than IAA or NAA indicated by greater number of explant forming callus. The use of 2,4-D as an auxin source for callus induction in legumes have also been reported in previous works in which 1-2 $mg.L^{-1}$ 2,4-D was the most effective (Kiran *et al.*, 2005; Kaviraj *et al.*, 2006; Kumari *et al.*, 2006; Ahlawat *et al.*, 2013). This can be understood since 2,4-D is categorized as a strong auxin having important role in stimulating the formation of callus under *in vitro* culture system.

Though the application of BAP or kinetin in combination with 2,4-D, IAA or NAA was important in promoting callus proliferation, the characteristic of callus regenerated from anthers of the two soybean cultivars, however, was the same. The colour of callus was white to greenish white and compact in structure. In other words, the effect of plant growth regulators showed no correlation to test soybean genotype. Similar effect of growth regulators was also reported by Zulkarnain *et al.* (2002) in anther culture of *Swainsona formosa,* a leguminous ornamental plant native to Australia.

The white colour and compact structure indicated that there was a embryogenic capacity of respected callus. This had been proven by a number of investigators, either on leguminous or non-leguminous plants. For example, in *in vitro* culture of *Bixa arellana,* Sha-Valli-Khan *et al.* (2002) found that the combination of NAA + BAP resulted in shiny white friable callus, that later turned white and compact before developing green globular structure. The development of such green globular structure indicated the early stage of embryogenesis as reported by Sudhersan and Abo-El Nil (2002) and Zulkarnain (2004) in *in vitro* culture of *Swainsona formosa.* The formation of white and compact callus that ended with embryogenesis was also reported by Fulzele and Satdive (2003) in tissue culture of *Nothapodytes foetida* and by Mohajer *et al.* (2012) in *Onobrychis sativa,* an important forage legumes.

Meanwhile, of the two tested soybean cultivars, it was found that Merubetiri was more responsive than Wilis under *in vitro* system. The effect of genotype on the *in vitro* culture of legumes had also been reported on *Cicer arietinum* (Arora and Chawla, 2005); Khan *et al.* (2011), *Phaseolus vulgaris* (Arias *et al.*, 2010) and *Cyamopsis tetragonoloba* and *C. serrata* Mathiyazhagan *et al.* (2013). In addition, variability in organogenic responses among genotypes was commonplace in numerous grain legumes including *Lathyrus sativus* (L.) (Ochatt *et al.*, 2007) and *Vigna subterranea* (Koné *et al.*, 2007; Koné *et al.*, 2013).

It is obvious that the response of explants during *in vitro* cul-ture is dependent upon the genotype of the donor plants. It is not only different species, but also different cultivars within species and, even individuals of the same cultivar that may show differences in embryogenic responses (Seguí-Simarro and Nuez, 2008a). Although the basis of genetic control is not understood yet, it was clear that genetic factors interacted with other factors to control the direction of explant development under *in vitro* culture system.

Based on reports on previous investigations, the regeneration of white and compact callus in this study implies a great opportunity to obtain haploid plants via embryogenesis from within callus mass that presumably consisted of haploid cells. By making some modifications on a number of environmental factors, particularly medium composition, it is hoped that the production of haploid soybeans via *in vitro* culture system could be realized, at least on Merubetiri that was more responsive than Wilis.

CONCLUSION

Based on the results obtain in this investigation, it can be concluded that:

1. The presence of plant growth regulators, particularly IAA, NAA or 2,4-D and BAP or kinetin, in culture medium was crucial in promoting callus formation on anther culture of soybean, either on Merubetiri or Wilis cultivars. However, Merubetiri was found to produce better *in vitro* response compared to Wilis.
2. Among tested auxins and cytokinins, the combination of 20 µM 2,4-D with 10 µM to 20 µM kinetin showed better results than other combinations.
3. Based on this reported investigation, future study should focus on *in vitro* culture of Merubetiri along with the use of 2,4-D and kinetin.

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