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Transformation of *Coffee arabica* using *chitinase* gene and regeneration of planlets from transformed-zygotic embryos

Transformasi Coffea arabica *menggunakan gen kitinase dan regenerasi planlet dari embrio zigotik-transforman*

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Ringkasan

Rekayasa genetika kopi arabika tahan penyakit cendawan dapat dilakukan dengan cara memasukkan gen kitinase (gen chi*) ke dalam genom tanaman tersebut. Penelitian ini bertujuan untuk mengintroduksikan gen* chi *pada kopi arabika serta meregenerasi eksplan yang ditransformasi menjadi plantlet. Gen* chi *disubkloning dari pBS G11 ke dalam plasmid pCAMBIA2301. Melalui* Agrobacterium tumefaciens*, plasmid rekombinan pCAMBIA2301/35s-chi kemudian dimasukkan ke dalam eksplan daun dan embrio zigotik kopi arabika. Eksplan daun transforman ditumbuhkan pada media seleksi yang mengandung kanamisin untuk induksi kalus embriogenik . Beberapa kombinasi 2,4-D dan dicamba serta kinetin, BAP dan 2-iP diuji kemampuannya untuk menginduksi terbentuknya kalus embriogenik. Embrio zigotik transforman ditumbuhkan pada media MS modifikasi yang mengandung kanamisin. Hasil penelitian menunjukkan bahwa perbedaan tipe sitokinin dan kombinasinya dengan 2,4-D atau dicamba. menyebabkan terjadinya variasi persentase pembentukan kalus embriogenik tahan kanamisin. Penambahan 100 mg/L kanamisin dalam media seleksi cukup efektif untuk menghambat pertumbuhan eksplan daun nontransforman. Persentase tertinggi induksi kalus embriogenik pada eksplan daun non transforman maupun transforman diperoleh pada media yang mengandung 5 μM 2,4- D dengan 5 μM of kinetin atau 5 mg/L dicamba dengan 5 μM BAP. Sedangkan dalam media dengan penambahan 5 μM kinetin, 100 mg/L asam sitrat dan 100 ppm asam askorbat, jumlah eksplan yang membentuk kalus mencapai optimum pada konsentrasi 0 dan 1 ppm dicamba untuk eksplan transforman dan 10 mg/L dicamba untuk non transforman. Pada eksplan embrio zigotik transforman, peningkatan konsentrasi kanamisin dari 100 mg/L hingga 500 ppm menurunkan persentase pengecambahan embrio dari 80.5 % menjadi 49%, persentase perakaran, dari 34 % menjadi 16%, jumlah akar, panjang akar dan tinggi tunas dari 7 mm menjadi 4 mm. Pada semua perlakuan kanamisin, embrio zigotik non transforman tidak membentuk akar dan pada umur kultur yang sama tunas yang dihasilkan lebih pendek dibandingkan dengan embrio-zigotik transforman. Hasil tersebut membuktikan bahwa gen ketahanan terhadap kanamisin* (NPTII) *telah terinsersi dan terekspresi dengan baik pada plantlet kopi arabika yang berasal dari eksplan embrio-zigotik transforman. Karena gen* chi *dikonstruksi dalam satu vektor dengan* NPTII*, maka diharapkan gen tersebut juga telah terinsersi ke dalam genom tanaman kopi.*

Summary

Genetic engineering of arabica coffee resistant to fungal diseases might be done by introducing a chitinase-encoding gene (*chi*) into genome of this plant. This research was aimed to introduce *chi* construct into arabica coffee and regenerate plantlets from the transformed explants. The *chi* gene was previously subcloned from pBS G11 into pCAMBIA2301 plasmid. With *Agrobacterium tumefaciens*, the recombinant plasmid pCAMBIA2301/35s-chi was then introduced into leaf and zygotic embryos explants of arabica coffee. The transformed leaf explants were cultured on the selection media containing kanamycin in the presence of several combinations of 2,4-D and dicamba with kinetin, BAP and 2-iP to induce the formation of embryogenic callus. The transformed zygotic embryos were cultured on the media of modified MS containing

kanamycin. The results showed that the several types of cytokinin used in combination with 2,4-D or dicamba caused the percentage of kanamycin resistant-embryogenic calli was varied. The addition of 100 mg/L kanamycin in the selection media was effective for inhibiting the growth of untransformed explants. Among the several combinations of auxin and cytokinin tested, the highest percentage of embryogenisis for untransformed and transformed leaf explants were achieved on the media containing 5 μM 2,4-D and 5 μM kinetin or 5 mg/L dicamba and 5 μM BAP. However in the presence of 5 μM kinetin together with antioxidants of 100 mg/L citric acid and 100 mg/L ascorbic acid, the explants calluses was optimum at 0 - 1 mg/L dicamba for transformed explants and 10 mg/L dicamba for untransformed explants. In the explants of transformed-zygotic embryos, increasing kanamycin from 100 mg/L up to 500 mg/L decreases the percentage of embryo germination from 80.5 % to 49%, rooted-shoots from 34 % to 16%, number of roots, root length and shoot length from 7 mm to 4 mm. At all the kanamycin treatments, root was not developed from the untransformed-zygotic embryos and the lenght of shoots were shorter compared to the transformed-zygotic embryos. This result demonstrates that the kanamycin-resistant gene (*NPTII*) has been inserted and well expressed in the plantlets of arabica coffee derived from transformed-zygotic embryos. Since the *chi* gene was constructed in one vector with *NPTII*, this gene might also been inserted in the genome of coffee.

[*Key words*: C*offee arabica*, chitinase gene, zygotic embryos, plant transformation]

Introduction

Genetic engineering of *C. arabika* for resistance to fungal diseases could be done by introducing a chitinase-encoding gene into the genome of this plant. Chitinases are one class of pathogenesis-related proteins, which have been implicated in plant defense against fungal infection by lysing the hyphal tips (Datta *et al.*, unpublished report). These enzymes catalyse the hydrolysis of β-1,4-linkage of N-acetylglucosamine polymer of chitin, a major component of fungal cell walls. Chitinases are widely found in several organisms, including bacteria (Jones, 1986), fungi (Yanai *et al.*, 1992; Siswanto & Darmono, 1998), and plant (Nishizawa & Hibi, 1991). Insertion of chitinases-encoding gene into plant genome has been reported for rice (Lin *et al.*, 1995), cucumber (Raharjo *et al.*, 1996) and tobacco (Terakawa *et al*., 1997). High-level expression of chitinase in transgenic plant can enhance resistance to a variety of fungal pathogens (Broglie *et al.*, 1991; Lin *et al.*, 1995). Introduction of this gene into cells of arabica coffee followed by plantlet regeneration in the selection media would result in transgenic plant of arabica coffee resistant to *H. vastatrix.*

Agrobacterium-mediated transformation has been applied to introduce specific gene into plant genome. This technique of gene transfer has been proven to be an important tool for genetic improvement. Productions of transgenic plant using *Agrobacterium tumefaciens* have been reported in several plant species including sweet orange (Pena *et al.*, 1995), cabbage (Jun *et al*., 1995) and cucumber (Raharjo *et al*., 1996). However, data for genetic transformation of coffee are limited. Barton *et al*. (1991) reported transformation of *C. arabica* protoplasts by electroporation but the protoplasts did not develop well. Transient expression of GUS gene on the surfaces of leaf tissue after biolistic delivery was reported by Van Boxtel *et al.* (1995), while Hatanaka *et al.* (1999) succeeded in regenerating transgenic coffee plants from embryogenic callus carrying GUS and selection marker genes of *HPT* and *NPTII.* Except for these reporter and selection marker genes, introduction of other agronomically important traits encoding genes has not been reported in coffee. This paper reports a progress of research work on geneticengineering of coffee resistant to *H. vastatrix* by introducing chitinase-encoding gene from rice into leaf explants and zygotic embryos of *C. arabica*.

Materials and Methods

Plant materials and sterilization procedure

Young leaf explants of *C. arabica* var. Kartika 1 and zygotic embryos of var. BP426A were used for transformation. Var. Kartika 1 was grown in the greenhouse, while var. BP426A was originated from collection garden of Indonesian Coffee and Cacao Research Institute, Jember, Indonesia.

Young leaf explants were prewashed and surface sterilized by 1 hour immersion in 1 g/L benlate solution, and then transferred into solution containing 5% Sodium hypochlorite for 30 minutes. After three rinses in sterile distilled water, the explants were cut (± 5x5 mm) and precultured on MS (Murashige & Skoog, 1962) basal media for aseptic prescreening. After 5-7 days cultured, the explants were used for transformation experiment.

Seeds were surface sterilized by overnight immersion in 0.1 g/L benlate solution, and then washed with sterile water. After peeling off the epidermal tissue, the seeds were then immersed again in 1 g/L benlate solution, followed by 5 minutes immersion in 95% ethanol and 45 minutes in 4% calsium hypochloride. The zygotic embryos were then collected aseptically from the seed in sterile condition.

Plasmid vectors and bacterial strains.

The recombinant plasmid vector of pCAMBIA2301/35s-*chi* carrying GUS reporter gene and *NPTII* selection marker gene was derived by insertion of *chi* fragment into pCAMBIA2301 (kindly provided by Dr. R.A. Jefferson, Australia) in the *Hin*dIII restriction site. The 35S- *chi* fragment was excised from pBS G11 (from Dr. Swapan Datta at IRRI, Philippines) by digestion with *Hin*dIII restriction enzyme. The recombinant plasmid of pCAMBIA 2301/35S-*chi* was transformed into *E. coli* JM109. Using *E. coli* S17, pCAMBIA-2301/35S-*chi* was then transferred into *A. tumefaciens* strain LBA4404.

The effect of kanamycin on the formation of embryogenic calli

To determine the minimal concentration of kanamycin that effectively inhibit the growth of leaf explant, kanamycin at several concentrations 0, 50, 100, 250, 500, 750 and 1000 mg/L was added into the medium. The composition of the medium was the same as that reported by Lopez-Baez *et al*. (1993).

Explants transformation procedure

Transformation experiments were carried out using *Agrobacterium. tumefaciens* strain LBA4404 harbouring recombinant plasmids of pCAMBIA/35S-chi. *Agrobacterium* was cultured on LB media containing 50 ppm kanamycin and incubated overnight at 28 ^{0}C on a shaker at 200 rpm in the dark. The bacterial suspension was diluted 1: 3 V/V with the same medium and grown for another 3 hours to bring the *Agrobacterium* concentration of about 10^9 cells/mL.

Transformation were carried out following the procedure as reported earlier (Siswanto, 2000) with little modification. For bacterial infection, leaf slices of Kartika 1 and zygotic embryos of BP 426A were incubated in *Agrobacterium*l suspension of about 10^9 cells/mL in the presence of 100 mg/L acetosyringone at 60 rpm for 15 min, 28 ^{0}C in the dark. The transformed leaf explants and zygotic embryos were then transferred to cocultivation medium, that was MS medium containing 100 mg/L acetosyringone for 2 days. The transformed leaves and zygotic embryos were rinsed three times using the same liquid media, with the last rinse containing 1250 mg/L claforan.

Induction of embryogenic callus from transformed-leaf explants

For embryogenic callus induction, the inoculated and washed leaf explants were cultured on the medium consisting of basal media ¼ strength macro salts dan ½ strength micro salts of MS medium (Murashige & Skoog, 1962), organic constituents of B5 medium, and 30 g/L sucrose (Hatanaka *et al*., 1991), with addition of 100 mg/L kanamycin and 250 mg/L claforan. This selection medium was supplemented with 5μM 2,4-D or 22.5 μM dicamba and 5 μM cytokinin BAP, 2-iP or kinetin respectively. The percentage of callus-forming explants was calculated at 4 and 6 weeks after transformation.

Regeneration of plantlets from transformed-zygotic embryos

For regeneration of transgenic plants from zygotic embryos, transformed-embryos were cultured in MS basal medium which has been modified by Priyono (1993) with addition of 100, 200, 300, 400, 500 mg/L kanamycin, respectively. The plantlets developed from optimum concentration of kanamycin were transferred to elongation medium, afterwards subcultured to the rooting medium (Priyono, 1993) supplemented with 100 mg/L kanamycin.

Results and Discussion

The effect of kanamycin on the formation of embryogenic callus

Table 1 shows the respond of leaf explants at various concentration of kanamycin in the selection media. In the media without kanamycin or the positive control, embryogenic calli were

Table 1. The effect of kanamycin on the percentage of explant survived with embryogenic callus initiation

Kanamycin (mg/L)	Percentage of explant survived (%) 2-week	3-week	4-week
0	100**)	100 (80**)	100 (80**)
50	100 (0)	100(9.2)	100 (9.2)
100	100(0)	75 (0)	45 (0)
250	83 (0)	48 (0)	32 (0)
500	57(0)	47 (0)	38 (0)
1000	0 *)	0	0

Note : 0) Explant were died ; **) percentage of embryogenic callus forming explants

formed directly from the edges of leaf cutting after 3 weeks in cultrure with the percentage of callus-forming explants of about 80%. The frequency of embryogenic callus initiation decreased sharply in the medium with kanamycin. During the first 4 weeks, the percentage of callus forming explants in the medium with 50 mg/L kanamycin was only 9.2%. At higher concentration of kanamycin 100-1000 mg/L, no callusing was detected and most of the explants become browning.

As shown in the Table 1, the effective concentration of kanamycin for totally killing the leaf explant was 1000 mg/L. However, addition of 100 mg/L kanamycin in the media was effective enough for inhibiting leaf growth. Therefore in the following transformation of leaf explants of *C. arabica,* concentration of kanamycin used in the selection medium was 100 mg/L.

One of the most important factors in regeneration of transgenic cells is the availability of an effective selection technique. Selection is important to inhibit growth of nontransformed cells and to enable transformants to survive and regenerate into complete transgenic plants. Otherwise, redundant of nontransformants will dominate the culture and result in chimeric plants (Parveez *et* al., 1996).

Kanamycin is one of the best-known antibiotics, which is widely applied as a selecting agent in transformation experiments. Resistance to kanamycin, geneticin G418, neomycin and paromomycin is obtained from the *aphA2* gene, also called *NPTII* (neomycin phosphotransferase II). This resistance occurs by phosphorylation of a specific hydroxyl group of these antibiotics, which results in failure of the antibiotics to bind to the ribosome (Wilmink & Dons, 1993). The effectivity of a selective agent was considered to be tissue and species specific (Colby & Meredith, 1990; Parveez *et al.*, 1996). Therefore, the effectivity of the selective agent should be determined before being used in transformation. Studies on five selective agents such as chlorsulfuron, glufosinate, glyphosate, hygromycin and kanamycin, indicated the potential of glufosinate for the detection of stably transformed coffee tissue (van Boxtel, 1994). Gimenez *et al*. (1994) attempted to establish a protocol for *C. arabica* transformation and evaluated three parameters: kanamycin resistance, transient expression of the gus gene (encoding ∃-glucuronidase) and level of infection of the *A. tumefaciens* strain (pTiC58). From the results obtained, the authors demonstrated that 100 mg/L of kanamycin inhibited callus growth, and that *Agrobacterium* induced a small infection detected on the basis of callus production in the tap root.

The uses of kanamycin for the selections of transformed cells have been published in many plant species. The finding of this study agrees with most of the published results. Generally, this antibiotic is used effectively for transformation of dicotyledonus tissues, but cannot easily be applied to monocot tissue (Colby & Meredith, 1990). For examples, concentrations more than 500 mg/L are needed to completely inhibit the growth of rice callus. At least 2500 mg/L

kanamycin was needed to fully inhibit the growth of oil palm embryogenic calli (Parveez *et al*., 1996). In contrast, only 50 mg/L of this antibiotic was needed in transformation of cucumber (Raharjo *et al*., 1996) and 15 mg/L for chinese cabbage transformation (Jun *et al*., 1995).

Induction of embryogenic callus from transformed-leaf explants

Several combinations of auxin and cytokinin affect the amount of explants to produce the embryogenic calli. Figure 1 shows that compared with two other treatments, combination of 5 µM 2,4-D and 5 µM of kinetin gives the highest percentage of callus-forming explants (24.6%). However, compared with the positive controls, in which untransformed-leaf explants cultured on the media without kanamycin, the percentages of transformed leaf explants producing embryogenic callus were much lower. These might be due to an inhibition effect of kanamycin. Number of callus-forming explants of positive control was also different for each type of cytokinin. The highest percentage (85%) was achieved on the media with 5 µM kinetin in combination with 5 µM 2,4-D. The number of callus-forming explants was significantly increased (40 – 77 %) when dicamba (22.5 µM) was used to substitute 2,4-D (Figure 2). The use of dicamba in combination with 5 µM BAP gave the highest percentage of callus formation. For all of the plant growth regulators combinations tested, the embryogenic calli grew slowly and after 4 weeks in the culture media, most of the explants and calli turned brown and died. In order to promote further development into somatic embryos, the embryogenic calli were subcultured into media with lower ratio of auxin/cytokinin. However, most of them were not developed. This might be due to browning effect that inhibits further development of embryogenic callus into somatic embryos.

Several reports on somatic embryogenesis of coffee have been published. Hatanaka *et al*. (1991) reported the effect of plant growth regulators on somatic embryogenesis in leaf cultures of *C. canephora*. They found that somatic embryogenesis was induced rapidly when cytokinin used as the sole plant regulator, and 5 µM was the optimum concentration for all kinds of cytokinin tested (2-iP, BA, and kinetin). Siswanto (2000) has reported formation of embryoid from leaf explants of *C. arabica* cultured in the induction medium containing 2,4-D in combination with BAP, 2-iP or kinetin. The highest percentage of embryoid-forming explants (63%) was achieved at combination of 10 µM 2,4-D with 5µM kinetin. Etienne-Berry *et al*. (1999) have succeeded in mass production of somatic embryos and plantlet regeneration of *C. arabica* on bioreactor.

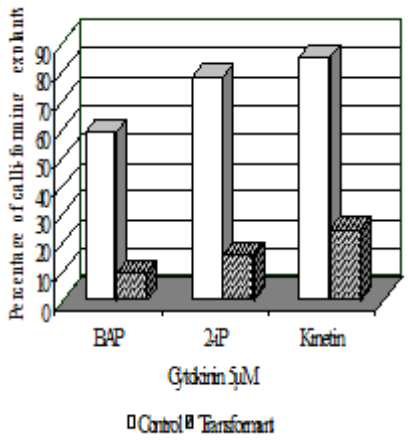

Figure 1. Number of embryogenic calli-forming leaf explants on the media containing 5 µM 2,4-D with different type of cytokinin.

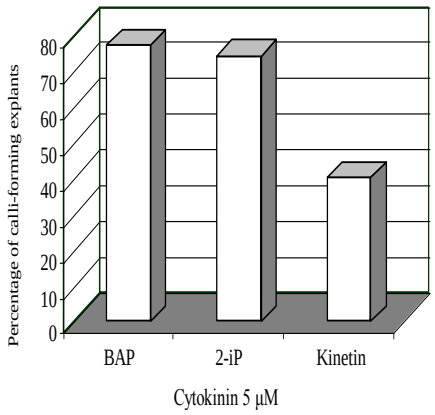

Figure 2. Number of callus-forming explants on the media containing 22.5 µM dicamba with different type of cytokinin.

To reduce browning of the leaf explants, the transformed and untransformed-leaf explants were cultured in the media containing dicamba (0 – 45 μM) and 5 μM kinetin with addition of 100 mg/L citric acid and 100 mg/L ascorbic acid as antioxidant. The results showed that the addition of antioxidant inhibited browning at least during 2 months of culture (Figure 3), which apparently promoted the growth of callus. After 3 months in the media containing only 5 μM kinetin , the explants calluses were about 80%. Addition of dicamba gradually decreased callus-forming explants from 78% to 20% (Table 2). In contrast to this, on the media without kanamycin, percentage of callus-forming untransformed explants (positive control) was higher if the concentration of dicamba increased.

Plantlet regeneration from transformed-zygotic embryos

The results showed that the concentration of kanamycin significantly affect the percentage of germination, rooting, root length and shoot height (Figure 4). In general, the higher concentration of kanamycin, the lower embryos germinate. As it is shown, both transformed and untransformed zygotic embryos germinate with different percentage. The highest percentage of germination (96%) was detected on the positive control that was untransformed-zygotic embryos cultured on the medium without addition of kanamycin. Transformed zygotic embryos germinate with the percentage of 48-80.5%. On the other hand, untransformed zygotic embryos cultured in the medium with kanamycin (negative controls) could germinate with lower percentage (25-55 %)

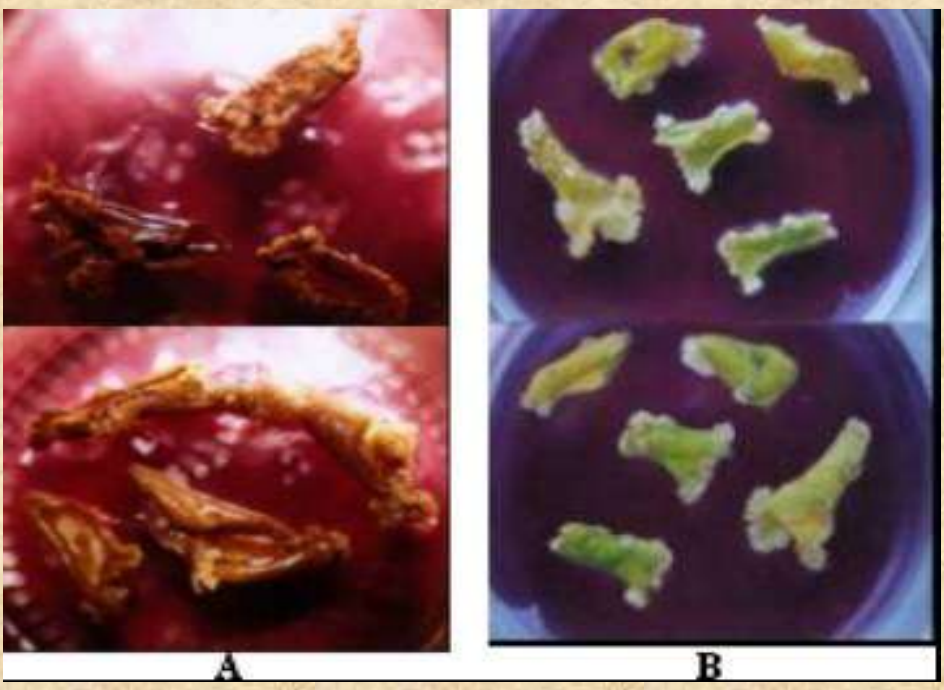

Figure 3. The appearance of embryogenic calli from the transformed-leaf explants at 1 month after transformation in the media (A)without and (B) with antioxidants of ascorbic acid and citric acids.

Table 2. Percentage of callus-forming explants on the medium with 5 μM kinetin in combination with dicamba and antioxidant citric acid and ascorbic acid

Dicamba (μM)	Transformed-explants			Untransformed-explants		
	40-day	3-month	Volume of callus	40-day	3-month	Volume of callus
	------- %	------		------- %	------	
0	14	80	1*)	23	42	1*)
4.5	53	78	2	26	39	3
11.25	41	50	2	45	74	3
22.5	35	58	2	60	87	4
45.0	20	20	2	50	92	5

Note : *) scoring for callus volume per explant, 1-5 (1-smallest; 5 - biggest) .

Table 3. The percentage of germinated transformed-zygotic embryos and plantlet growth on the selection medium with different concentration of kanamycin.

Control/ Transforman	Kanamycin (mg/)	Germinated embryos (%)	Plantlet*) (%)	No. of root per plantlet	Root Length (mm)	Shoot Length (mm)
Control (+)	0	96	94	1-3	3-10	10
Control (-)	100	(55	0	0	0	4
Control (-)	300	25	0	0	0	3
Control(-)	500	25	0	0	0	2
Transforman	100	80.5	34	1-3	5-6	6.7
Control (-)	200	35%	0	0	0	4
Transforman	200	58%	43	1-2	3-4	7
Transforman	300	48%	31.3	1	2	6
Transforman	500	49%	16	1	1	4

Note: *) Planlet with root, **) Numbers of germinated embryos

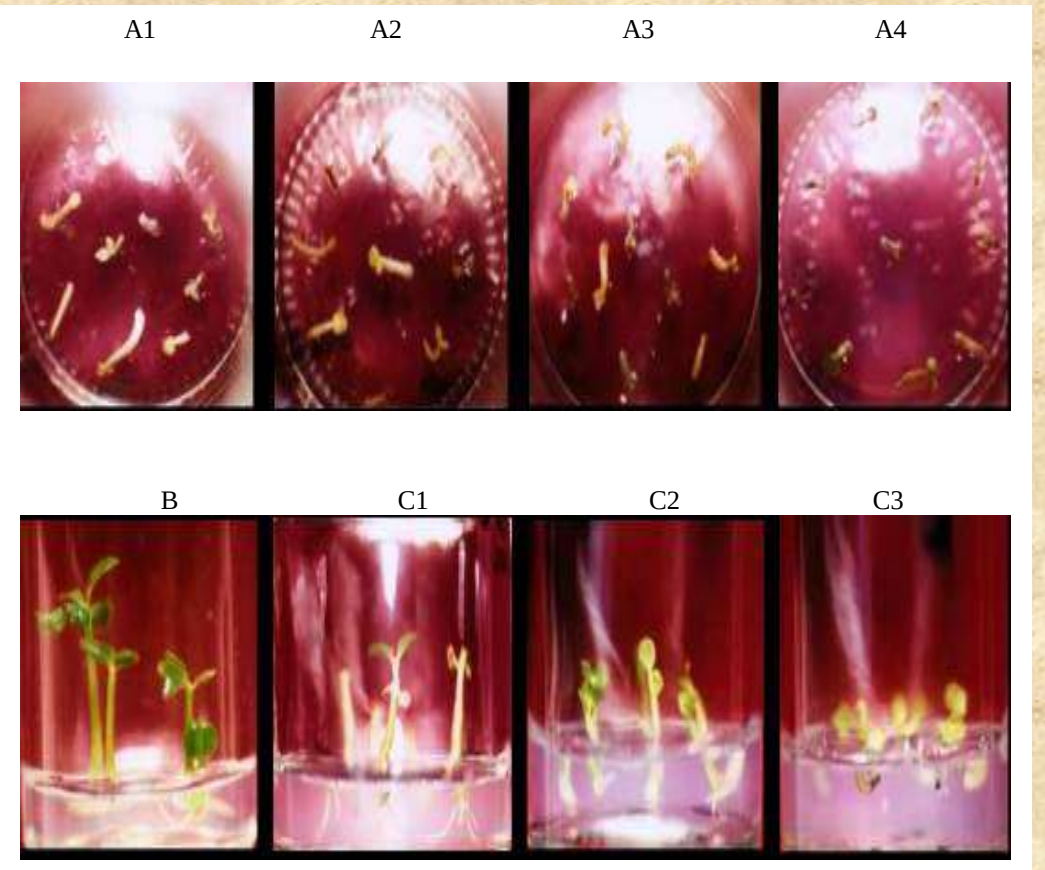

Figure 4. Development of (A) untransformed; (A1) & (C1) - 100 mg/L kanamycin; (A2) & (C2)- 200 mg/L kanamycin; (A3) - 300 mg/ L kanamycin; (A4) & (C3)-500 mg/L kanamycin and (C) transformed, zygotic embryos of *C. arabica* on the medium with several concentrations of kanamycin in comparison with (B) positive control- 0 mg/L kanamycin.

Except for the negative control, germinated embryos developed into plantlet. The percentage of plantlet-developing embryos for the positive control was 94%, much higher compared with that of transforman 16 – 43%. On the other hand, no root developed from untransformed zygotic embryos (negative controls). This indicates that kanamycin at concentration range of 100 – 500 mg/L effectively inhibit development of zygotic embryos into plantlet. Number of root per shoot, length of root and shoot height were affected by concentration of kanamycin.

The higher the kanamycin concentration, the lower the root number and length and shoot height. These again indicate the inhibition of root development by kanamycin. These results showed that the rooted shoots (plantlets) developed from transformed zygotic embryos were kanamycin resistant. Since kanamycin resistant gene (*nptII*) was constructed in one vector with *chi* gene, the *chi* gene might also been inserted in the genome of coffee.

The presence of kanamycin resistant gene and *chi* gene in the embryogenic calli of transformed-leaf explants and in the plantlets regenerated from transformed zygotic embryos should be further analyzed by polymerase chain reaction (PCR) using specific primers.

Conclusions

Kanamycin at 100 mg/L was sufficiently effective to inhibit both callus induction from untransformed-leaf explants and plantlet regeneration from untransformed-zygotic embryos.

The highest percentage of embryogenic calli induction from transformed leaf explants was achieved on the media containing 22.5 μM dicamba and 5 μM BAP.

Addition of antioxidants citric acid and ascorbic acid prevented browning of the embryogenic calli, but no further development detected from these calli. In the selection media, transformed-zygotic embryos developed into plantlets.

The percentage of germination, rooting, root length, and shoot length was lower compared to that from the untransformed-zygotic embryos (positive control).

These results demonstrated that the kanamycin resistant gene might had been inserted into the genome and expressed both in the embryogenic calli and in the plantlets.

References

Barton, C.R., T.L. Adams, M.A. Zarowitz (1991) Stable transformation of foreign DNA into *Coffea arabica* plants. *In*: Proceedings of 14th Colloquium of International Coffee Science Association (ASIC), San Francisco, USA. pp. 460-464.

Broglie K., I. Chet, M. Holliday, R. Cressman, P. Biddle, S. Knowlton, C.J. Mauvanis & R. Broglie (1991). Transgenic plants with enhanced resistance to fungal pathogen *Rizoctonia solani*. *Science*, **254**, 1194 – 1197.

Colby, S.M. & C.P. Meredith (1990). Kanamycin sensitivity of cultured tissues of *Vitis*. *Plant Cell Rep.*, **9**, 237-240.

Datta, K., J.Tu, N. Oliva, S. Muthukrisnan & S.K. Datta. *Transgenic rice for enhance resistence to* Rizoctonia solani *causing sheath blight disease*. Manila, Plant Breeding, Genetic and Biochemistry Division, PO Box 933. Unpublished report.

Etienne- Berry, D., B. Bertrand, N. Vasquez & H. Etienne (1999). Direct sowing of *Coffea arabica* somatic embryos mass produced in a bioreactor and regeneration of plants. *Plant Cell Rep.*, **19**, 111-117.

Gimenez, C., A. Menendez-Yuffa, E. Garcia. (1994) Preliminary studies for genetic improvement of coffee (*Coffea arabica*) by methods of genetic engineering. *In*: Abstracts of the VIIIth International Congress of Plant Tissue and Cell Culture, Florence, Italy, June 12- 17. p. 163.

Hatanaka, T., O. Arakawa, T. Yasuda, N. Uchida & T. Yamaguchi (1991). Effect of plant growth regulators on somatic embryogenesis in leaf cultures of *Coffea canephora*. *Plant Cell Rep.*, **10**, 179-182.

Hatanaka, T., Y. E. Choi, T. Kusano & H. Sano (1999). Transgenic plants of Coffee *Coffea canephora* from embryogenic callus via *Agrobacterium tumefaciens*-mediated transformation. *Plant Cell Rep.*, **19**, 106-110.

Jones, J.D.G. (1986). Isolation and characterization of genes encoding two chitinase enzymes from *Serratia marcescens*. *EMBO J.*, **5**, 467-473.

Lin, W., C.S. Anuratha, K. Datta, I. Potrykus, Mathukrisnan & S.K. Datta (1995). Genetic engineering of rice for resistance to sheath blight. *Iotechnology*, **13**, 686 – 691.

Murashige, T., & F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.*, **15**, 473-497.

Jun, S.I., S.Y. Kwon, K.Y. Paek & K-H Paek (1995). *Agrobacterium*-mediated transformation and regeneration of fertile transgenic plants of chinese cabbage (*Brassica campestris* spp. *Pekinensis* cv. 'spring flavor'). *Plant Cell Rep.*, **14**, 620-625.

Nishizawa, Y. & T. Hibi (1991). Rice chitinase gene: cDNA cloning and stress-induced expression. *Plant Sci.* **76**: 211-218.

Parveez, G.K.A., M.K.U. Chowdhury & N.M. Saleh (1996). Determination of minimal inhibitory concentration of selection agents for oil palm (*Elaeis guineensis* Jacq.) Transformation. *Asia Pacific J Mol. Biol. Biotech*, **4,** 219-228.

Pena, L., M. Cervera, J. Juarez, A. Navarro, J.A. Pina, N. Duran-Vila & L. Navarro (1995). *Agrobacterium*-mediated transformation of sweet orange and regeneration of transgenic plants. *Plant Cell Rep.* **14**, 616-619.

Priyono (1993). Embryogenesis somatic langsung pada kultur *in vitro* eksplan daun kopi arabika (*Coffee arabica*). *Jurnal Ilmiah Pertanian Indonesia*, **3,** 1-10.

Priyono, B. Purwadi, A.W. Susilo, S. Mawardi (2000). Frekuensi terbentuknya biji poliembrioni pada beberapa klon kopi robusta dan keberhasilannya pada kultur *in vitro*. *Pelita Perkebunan*, **16**(3), 110-120.

Raharjo, S.H.T., M.O. Hernandez, Y.Y. Zhang & Z.K. Punja (1996). Transformation of Pikling Cucumber with Chitinase-encoding Genes using *Agrobacterium tumefaciens*. *Plant Cell Rep***. 15,** 591 596.

Siswanto & T.W. Darmono (1998). Chitinase and β-glucanase activities against *Ganoderma sp*. In Oil palm. *In:* Proc. BTIG Workshop on oil palm Improvement through Biotechnology, Bogor 16-17 April 1997. p. 104-114.

Siswanto (2000). Perakitan tanaman transgenik kopi arabika tahan terhadap penyakit karat daun. Laporan Akhir RUT VII Tahun 2000.

Terakawa, T. , N. Takaya, H. Horiuchi & M. Koike (1997). A fungal chitinase gene from *Rhizopus oligosporus* confer antifungal activity to transgenic tobacco. *Plant Cell Rep.* **16,** 439 – 443.

Van Boxtel, J. (1994) Studies on genetic transformation of coffee by using electroporation and the biolistic method. *Doctoral thesis, Wageninge*n. 125 pp.

Van Boxtel, J., M. Berthouly, C. Carasco, M. Dufour & A. Eskes (1995). Transient expression of β-glucuronidase following biolistic delivery of foreign DNA into coffee tissues. *Plant Cell Rep*., **14,** 748-752.

Wilmink, A. & J.J.M. Dons (1993). Selective agents and marker genes for use in transformation of monocotyledonous plants. *Plant Mol. Biol. Rep.*, **11**, 165-185.

Yanai, K., N. Tanaka, N. Kojima, H. Horiuchi, A. Ochta & M. Takago (1992). Purification of two chitinases from *Rhizopus oligosporus* and isolation and sequencing of the encoding genes. *J. Bacteriol.*, **174**, 7398-7406.