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Research Article
An Efficient Protocol for *Agrobacterium*-mediated Transformation of Sugarcane by Optimizing of Duration of Co-cultivation and Age of Callus

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Abstract

Background and Objective: Concentration of sucrose in sugarcane was affected by several enzymes, one of which is pyrophosphate-dependent phosphofructokinase (PFP). The PFP is an enzyme that catalyzed the conversion of fructose 6-phosphate (F6P) and pyrophosphate (PPi) to fructose 1,6 bisphosphate and inorganic phosphate (Pi) reversibly. The PFP activity is inversely correlated to sucrose content in sugarcane. In this study, insert RNAi *pfp* gene to inhibit PFP protein so, sucrose content in sugarcane increased. The study objective is to obtain an efficient protocol of *Agrobacterium*-mediated transformation of sugarcane was needed. **Materials and Methods:** This study used *A. tumefaciens* strain GV3101 containing expression vector of pART27. Calluses from sugarcane cv. Bululawang (BL) and cv. PS862 were used as explants. Callus induction of sugarcane leaf roll was performed in solid MS medium supplemented with 3 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. Treatments of co-cultivation were 24 and 72 h whereas, age of callus was 7th and 10th weeks old. Selection was done in MS medium supplemented with kanamycin 100 mg L^{-1}. Planlets which have kanamycin resistant were analyzed by PCR. **Results:** The study results showed that the highest transformation efficiency was obtained from treatment of co-cultivation 72 h and 7th weeks old of callus whereas, 7th weeks old of callus as an target for transformation have better transformation efficiency than 10th weeks old. Many planlets show the positive PCR. **Conclusion:** Co-cultivation duration and difference age of callus affected the success of RNAi *pfp* gene transformation of sugarcane mediated by *A. tumefaciens*. An efficient protocol was obtained at use of co-cultivation 72 h and 7th weeks old callus.

Key words: *Agrobacterium tumefaciens*, co-cultivation, RNAi *pfp* gene, sugarcane, transformation, kanamycin, acetoryringone, PCR

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Sugarcane (*Saccharum officinarum*) is a highly economic value because it was used as a raw material of sugar. The increasing number of population in step with the increasing demand of sugar but it is not accompanied by an increase sugar production. Concentration of sucrose in sugarcane was affected by several enzymes, one of which is pyrophosphate-dependent phosphofructokinase (PFP). The PFP is an enzyme that catalyzed the conversion of fructose 6-phosphate (F6P) and pyrophosphate (PPi) to fructose 1,6 bisphosphate and inorganic phosphate (Pi) reversibly. The PFP activity is inversely correlated to sucrose content in sugarcane. It can be seen from the fact that the increase in sucrose content will be followed by decrease of PFP activity[1]. Sucrose content in sugarcane is also affected by other enzymes including invertase[2] (EC 3.2.1.26), sucrose phosphate synthase[3] (EC 2.3.1.1) and sucrose synthase[4] (EC 2.4.1.13). Several studies using gene transformation technique to increase levels of sucrose in sugarcane were done by lowering the activity of invertase enzyme and by silencing *pfp* gene[5]. In this study, insert RNAi *pfp* gene to inhibit PFP protein so, sucrose content in sugarcane increased. The study objective is to obtain an efficient protocol of *Agrobacterium*-mediated transformation of sugarcane was needed.

The successful transformation technique is influenced by several factors including explant type or explant age, the presence or absence of wounding on the explants and co-cultivation duration. Many studies to improve transformation efficiency by co-cultivation duration has been done on several crops, namely rice to improve GUS gene expression[6] and orange[7]. This study will conduct transformation of RNAi *pfp* gene in sugarcane mediated *A. tumefaciens* by optimizing the duration of co-cultivation and age of callus as an explants. The successful transformation of RNAi *pfp* gene in to sugarcane plant was expected to increase sugar yield and will give contributed to plant breeding.

MATERIALS AND METHODS

Sugarcane cv. Bululawang (BL) and cv. PS 862 were obtained from PT. Perkebunan Nusantara XI, Surabaya, Indonesia. *Agrobacterium tumefaciens* strain GV 3101 containing plasmid pART27 was obtained from Laboratory of Chromosomes and Molecular Biology Analysis-Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung. Plasmid pART27 contains RNAi *pfp* gene, neomicin phosfotransferase II (*npt* II) gene with a cauliflower mosaic virus (CaMV) promoter as presented in Fig. 1.

Callus was induced from sugarcane leaf rolls. Leaf roll is located between the first and second leaf with length 20 cm and diameter 1.5 cm. Leaf was sterilized by sprayed with 70% alcohol and then was burned. Leaf roll was peeled layer by

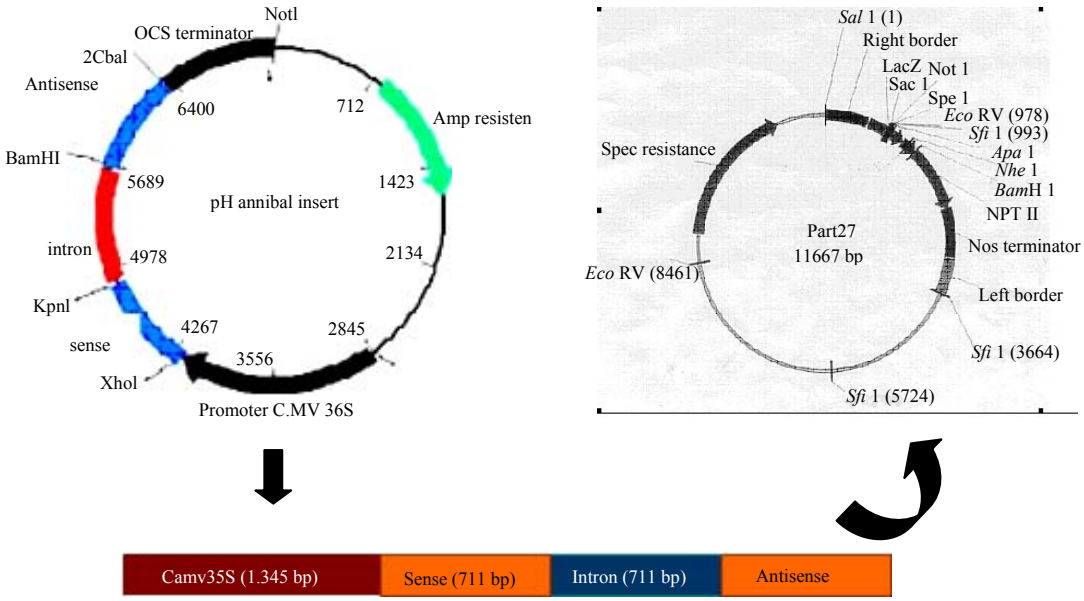

Fig. 1: Construct of RNAi *pfp* gene on plasmid pART27

layer until the stalk diameter around±0.5 cm then the innermost leaf roll was excised cross-sectional in about 2-3 mm starting 2.0 cm from the node towards the top. Small pieces of leaf roll were planted in MS (Murashige and Skoog) medium supplemented with 3 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin and then incubated in the dark for 8 weeks with one time subculture at the age of 4 weeks.

Agrobacterium tumefaciens strain GV 3101 was grown on YEP solid medium containing 20 mg L^{-1} spectinomycine and 50 mg L^{-1} riphamycine at 28°C for 3 days. One colony of *Agrobacterium* was taken and grown in YEP liquid medium and was grown at 28°C and shaking 150 rpm for 15 h. Bacterial density was measured at $OD_{600} = 0.8$. Eight weeks old callus were cut±0.1 cm^2 and soaked in 20 mL of liquid MS medium supplemented with 2 mL of *A. tumefaciens* for 30 min. Callus was grown on co-cultivation medium (MS containing 100 μM acetosyringone) for 24 and 72 h. Each treatment was incubated in the dark at 28°C. After co-cultivation stage, callus was grown on MS medium supplemented with 500 mg L^{-1} cefotaxime for 7 days. After that, callus was grown on MS medium containing 0.5 mg L^{-1} kinetin and 2 mg L^{-1} IAA supplemented with 100 mg L^{-1} kanamycin for 6 weeks at 25°C in the light conditions. Callus were subculture in 3rd week. Kanamycin-resistant callus were then induced to shoots regeneration in MS medium supplemented with 2.0 mg L^{-1} IAA without antibiotics for 6 weeks with only one subculture.

Treatment of different age of callus was done by used 7th and 10th weeks old callus. Plantlets then were analyzed by PCR.

The DNA isolation was done on sugarcane plantlets which was kanamycin resistant (Qiagen). The PCR was performed with RNAi *pfp* gene (nucleotide sequence are: Foward 5'-GGA AGA AAC CCT AAA ACT CGT CGC-3', reverse 5'-GAA TTC ACG TGC TGT TCC TGT CAA AAC ACC AGA-3'). The PCR reactions used the PCR master mix (Roche) with a final volume of 25 μL. The condition for PCR was pre-denaturation (95°C for 4 min), denaturation (95°C for 30 sec), annealing (57°C for 40 sec), elongation (72°C for 60 sec) and the final elongation for 7 min. Amplified DNA was analyzed using 1% agarose gel electrophoresis at 100 V for 20-25 min and visualization was used ethidium bromide staining.

RESULTS

Shoots regeneration of sugarcane cv. BL after treatment co-cultivation 72 h was 39 shoots with a transformation efficiency 13% while, co-cultivation 24 h, it did not any shoots. Whereas, the number of shoot of sugarcane cv. PS 862 after treatment co-cultivation 72 h were 10 shoots but the others were yellow color (chlorotic) so that the transformation efficiency was 5.5%. Co-cultivation 24 h have only one shoot which was able to regenerated so that the transformation efficiency obtained was 0.6% (Table 1). Development stages of sugarcane cv. BL and cv. PS 862 after *Agrobacterium*-mediated transformation are presented in Fig. 2.

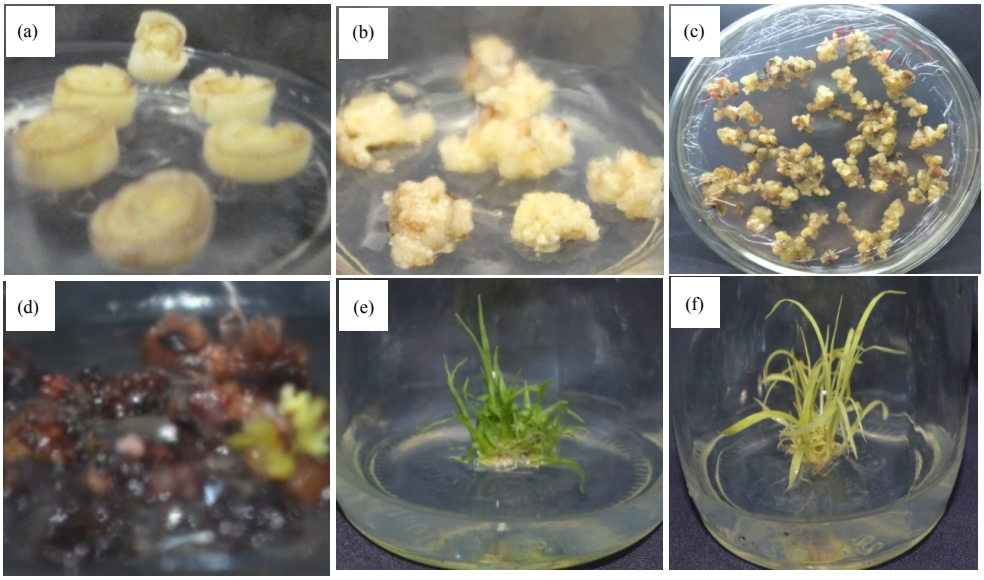

Fig. 2(a-f): *Agrobacterium*-mediated transformation of sugarcane cv. BL and cv. PS 862, (a) Leaf roll explants, (b) Eight weeks old of callus, (c) Callus in co-cultivation medium, (d) Callus in selection medium (e) Kanamycin-resistant planlets of sugarcane cv. BL and (f) cv. PS 862

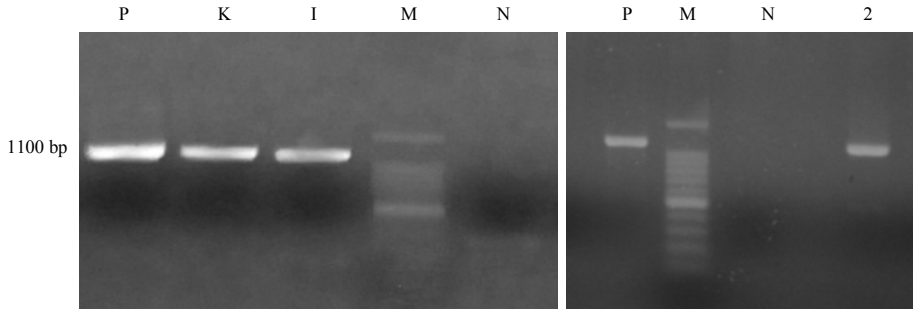

Fig. 3: PCR analysis of kanamycin resistant planlets, P: Positive control (plasmid pART27 contains RNAi *pfp* gene), K: Positive kontrol (transformant plant of T1 generation of sugarcane cv. KK), 1: Transformed planlet sugarcane cv. BL, 2: Transformed planlet sugarcane cv. PS 862 and N: Negatif control (wild type)

Table 1: Effect of co-cultivation on transformation efficiency of sugarcane cv. BL and PS 862

	No. of calli		No. of shoots on selection medium		No. of shoots on regeneration medium		Transformation efficiency (%)*	
Sugarcane cultivars	K1	K2	K1	K2	K1	K2	K1	K2
BL	221	292	0	42	0	39	0	13
PS862	156	181	66	21	1	10	0.6	5.5

K1: Co-cultivation 24 h, K2: Co-cultivation 72 h and *No. of shoots on regeneration medium/No. of calli×100

Table 2: Effect of age of callus on transformation efficiency of sugarcane cv. PS 862

	No. of calli		No. of shoots on selection medium		No. of shoots on regeneration medium		Transformation efficiency (%)*	
Sugarcane cultivars	K1	K2	K1	K2	K1	K2	K1	K2
PS862	220	319	90	125	17	15	7.7	4.7

A1: Seven weeks old callus, A2: Ten weeks old callus and *No. of shoots on regeneration medium/No. of calli×100

Morphologically, kanamycin resistant plantlets of sugarcane cv. PS 862 showed yellow and green leaves. The difference in leaves color was assumed the random insertion of the gene that caused changes in chlorophyll formation. In addition, it was also caused by the place of the introduction of genes. If the gene inserted in the chloroplast and genes encoding chlorophyll formation became impaired. Kanamycin-resistant plantlets of sugarcane was assumed have been inserted by RNAi *pfp* gene but to support this results PCR analysis was needed.

Confirmation test on the presence of RNAi *pfp* gene using PCR method resulted one kanamycin-resistant plantlet of cv. BL and cv. PS 862 have positive PCR, respectively. This can be proven by the 1,110 bp expected RNAi *pfp* gene which was found same as the positive control (plasmid pART27 contains RNAi *pfp* gene) (Fig. 3). Although, plantlets passed in the selection medium not all plantlets showed a positive PCR. This was possible because the plantlets escaped or avoided the selection.

Ninety transformant (40%) callus of sugarcane cv. PS 862 which originated from 7th weeks old callus could grew on the selection medium. Its showed that callus have kanamycin resistant. After subculture in the medium without antibiotic, callus developed to 17 putative transformed planlets. Whereas, 125 (39%) callus which originated from 10th weeks old grew on the selection medium and regenerated to 15 putative transformed planlet (Table 2). In Fig. 4 showed the regeneration stage of callus to putative transformed planlet. Putative transformed planlets at 10th weeks old have various color, there are green yellowish (87.5%) and dan green (12.5%).

In this study, non transformed calli showed necrosis. Calli grew relatively slow, small proliferation was observed on the surface of the calli that had turn brown to black color but some of calli regenerated shoots with white color (Fig. 4). The PCR products showed a band at 1,110 bp similar to the positive control (Fig. 5).

DISCUSSION

Effect of co-cultivation on transformation efficiency: Duration of co-cultivation was very important because it

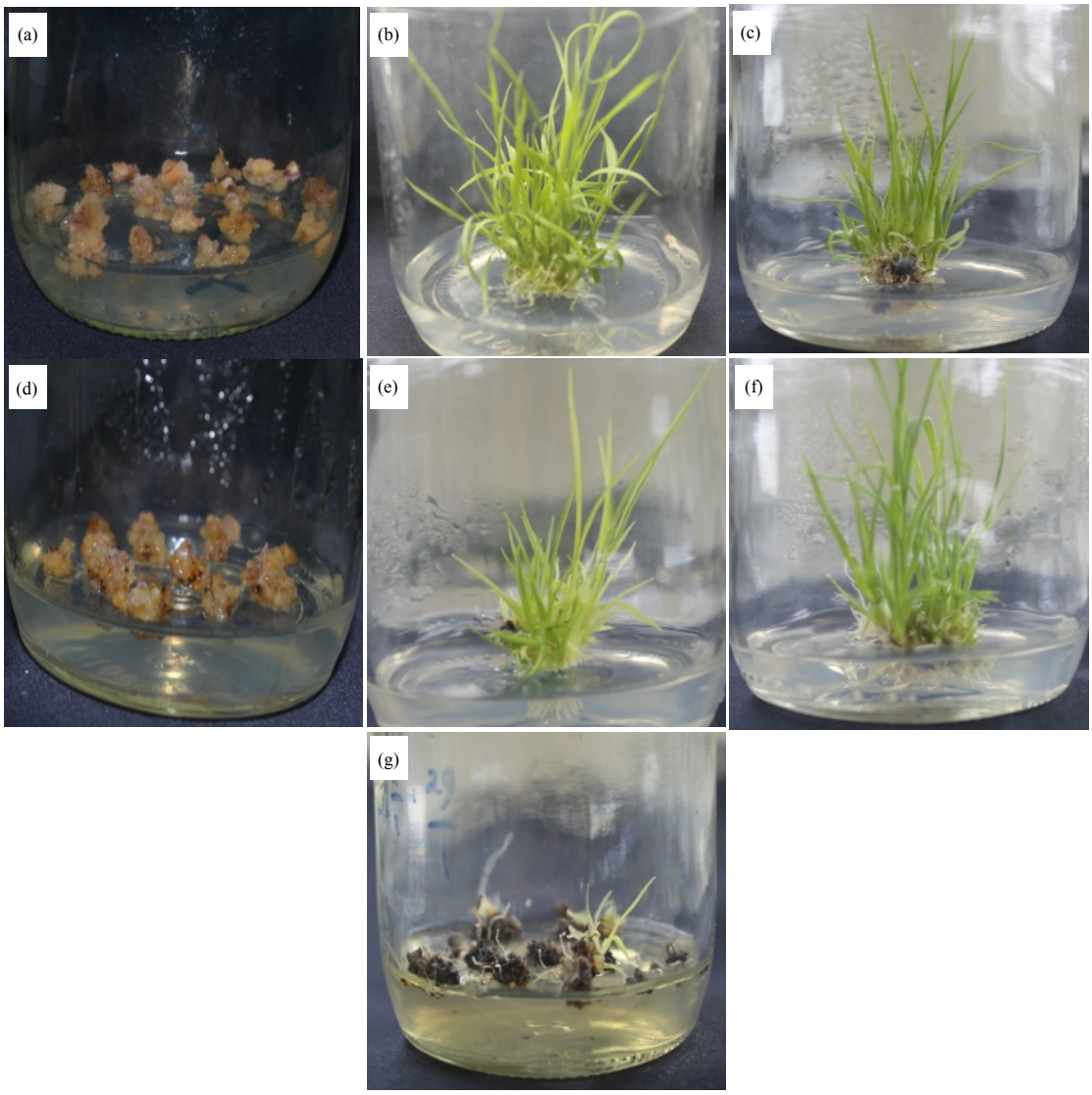

Fig. 4(a-g): Developmnet of transgenic plant which originated from 7th and 10th weeks old callus, (a) Six weeks old callus, (b-d) Putative transformed planlets originated from 6th weeks callus, (e-f) Difference colour of putative transformed planlet originated from 10th weeks callus and (g) Non transformed callus

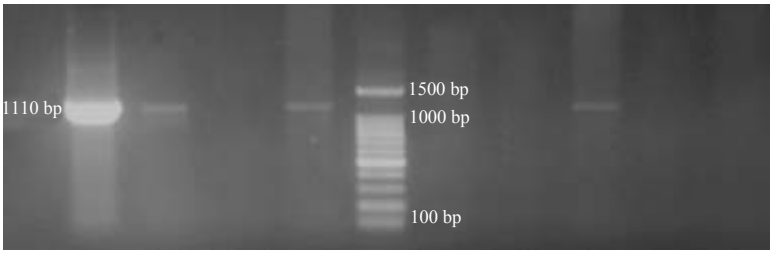

Fig. 5: PCR analysis of putative transformed planlet, M: Molecular weight, P: Positive control (plasmid pART27 contains RNAi *pfp* gene), N: Negative control (wild type), Lane 1-4: Tranformed planlet which originated from 7th weeks old callus and Lane 5-7: Transformed planlet which originated from 10th weeks old callus

affected the effectiveness of *A. tumefaciens* bacterial infection. If co-cultivation very quickly, it made bacteria not be able to infect the cells perfectly but co-cultivation long time there would be bacterial overgrowth so that inhibited the growth and even killed the explants. This was due to the level of competition between the explants and bacteria in utilizing the nutrients and it resulted in the inhibition of explants growth. Therefore, the transformation process required appropriate co-cultivation duration.

In this study, duration of co-cultivation 72 h have higher transformation efficiency than 24 h. The use of co-cultivation 72 h has been performed on the *Agrobacterium*-mediated transformation of sugarcane[8] and some plants including indica rice[9], cabbage[10], rice[11,12], pineapple[13] and *Jatropa curcas*[14].

Morphologically, kanamycin resistant plantlets of sugarcane cv. PS 862 showed yellow and green leaves. The difference in leaves color was assumed the random insertion of the gene that caused changes in chlorophyll formation. In addition, it was also caused by the place of the introduction of genes. If the gene inserted in the chloroplast so that genes encoding chlorophyll formation became impaired. Kanamycin-resistant plantlets of sugarcane was assumed have been inserted by RNAi *pfp* gene but to support this results PCR analysis was needed.

Callus of sugarcane cv. PS 862 which originated from 7th weeks old callus could grew on the selection medium. Its showed that callushave kanamycin resistant. After subculture in the medium without antibiotic, callus developed to putative transformed planlets. Whereas, callus which originated from 10th weeks old grew on the selection medium and regenerated to putative transformed planlet. Putative transformed planlets at 10th weeks old have various color, there are green yellowish dan green. It was assumed that planlets were influenced by kanamycin. Negative effect of kanamycin have been reported on growth and development of transgenic banana which was showed albino and semi-necrotic planlets[15]. Similar result also happened in pea (*Pisum sativum* L.)[16]. Kanamycin as a selection agent could effect of mitochondria and chloroplast by destructed the protein synthesis so, the plants became chlorosis[17]. In this study, non transformed calli showed necrosis. Calli grew relatively slow, small proliferation was observed on the surface of the calli that had turn brown to black colour but some of calli showed growth and regenerated the shoot with white color. The necrosis and browning of the tissue after pathogen infection are likely defense responses[18,19].

Effect of callus age on transformation efficiency: Difference of the explants age will influence of regeneration of the transformant. Many studies showed that callus could grow in the selection medium but not all of callus could regenerated to planlet. Transformation efficiency of sugarcane cv. PS 862 which was originated from 7th weeks old callus higher than 10th weeks old callus. It was assumed that the age of explants and viability callus influenced of regenerated to induce planlets. Meanwhile, target of transformation mediated *Agrobacterium tumefaciens* should from embriogenic tissue because its cells have activity mitotic fast so the probability integration of T-DNA to plant genome higher[20]. Beside that, transfer and integration of T-DNA also depend on cell cycle of host and it happened during DNA replication[21]. Transformation efficiency of Italian ryegrass (*Lolium multiflorum* Lam.) cv. Hwasan 101 was 6.7% after co-cultivation 5 days and 9th weeks old callus. *Agrobacterium tumefaciens* mediated genetic transformation of *Lycium barbarum* L. which used 3 weeks old of seedling as an explants have transformation efficiency higher than 4-5 weeks old of seedling[21]. Regeneration of wheat immature embryo was highly related to the environment conditions and the age of donor plants[22-25].

Putative transformant of sugarcane cv. PS 862 which was kanamycin resistant and positive PCR formed two clumps (originated from 7th weeks old callus) and one clump (originated from 10th weeks old callus). Whereas, 19 clumps which was analyzed by using PCR have no RNAi *pfp* gene amplification. It showed that those of kanamycine resistant planlet were non transformed (escape). The high number of escape could be caused by the big size of initial explants so, some of the explants could not exposed to the selective agent present in the culture medium consequently, the selection efficiency was low.

CONCLUSION

The efficient protocol of *Agrobacterium*-mediated transformation of sugarcane have successful achieved at co-cultivation duration and difference age of callus. The best transformation efficiency was obtained from treatment of co-cultivation 72 h and 7th weeks old of callus. It could provide opportunities at the next study to develop sugarcane which has ability to produce high yield of sugar.

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