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THE METHOD OF *Agrobacterium Tumefaciens*-MEDIATED *MmCu/Zn-SOD* GENE TRANSFORMATION IN THE RED SEAWEED *Kappaphycus alvarezii*

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ABSTRACT

Superoxide dismutase (*SOD*) has an important role in the defense system of the body, especially the activity of reactive oxygen compounds that can cause stress. *MmCu/Zn-SOD* gene successfully regenerated in *N. benthamiana* and *N. tabacum,* rice, and Jatropha transgenic plants. The assembling of transgenic *K. alvarezii* has been previously performed with other genes, however, the successful introduction of genes was still low. The objective of this study was to optimize a method for the *Agrobacterium* mediated *MmCu/Zn-SOD* gene transformation based on the optical density (OD), the duration of inoculation and co-cultivation period factors to increase the effectiveness of transgenesis in *K. alvarezii*. The experimental method was divided into the two stages: 1) the optimization of *MmCu/Zn-SOD* gene transformation in *Kappaphycus alvarezii* based on optical density (OD), inoculation duration and co-cultivation period; and 2) the analysis of the existence of *MmCu/Zn-SOD* genes in *Kappaphycus alvarezii* using PCR. Six levels of *A. tumefaciens* OD_{600} (0.3, 0.4, 0.5, 0.6, 0.7 and 0.8), four duration of inoculation (10, 20, 30 and 60minutes) and two periods of co-cultivation (3 and 4days) were tested.

The results demonstrated that the highest putative bud (100%) and transformation (100%) efficiencies were obtained by using inoculation duration of 60minutes and 3days of co-cultivation with OD_{600} of 0.5. However, regeneration efficiency of explants (6.67%) was lower than the non-transgenic control (33.33%). The results of PCR analyses demonstrated the presence of *MmCu/Zn-SOD* gene in the transgenic explants, whereas the non-transgenic explants seaweed showed no amplification product. In conclusion, transgenic *K. alvarezii* could be produced and optimized by the *Agrobacterium tumefaciens*-mediated method based on optical density (OD), duration of inoculation and co-cultivation period factors. Transgenic explants that carry encoding genes of *MmCu/ Zn-SOD* tolerant to salinity of 15g /L and salinity of 45g/L so all were survive, while all the non-transgenic were died.

Keywords : *Agrobacterium tumefaciens*, method, *MmCu/Zn*-SOD gene, *Kappaphycus alvarezii*, transformation.

1. INTRODUCTION

Seaweed is a coastal resource with a high economic value. It has several advantages including a high export market opportunity, short maintenance periods and can supply a variety of processed products. A serious problem of the red seaweed *Kappaphycus alvarezii* farming is declining yields because of ice-ice disease (white spots), which has caused decrease in seaweed production by roughly 70-100% (Largo *et al*., (1995); Vairappan *et al*., (2008). Ice-ice disease is mainly caused by extreme environmental changes over time. Seaweed is experiencing stress due to changes in environ-mental conditions such as changes in salinity, water temperature, light intensity and pH. The pathogen is easier to infect and it is the main factor the emergence of ice-ice disease (Arisandi *et al*., 2011). The introduction of SOD genes through transgenic techniques may create a red seaweed that is stress-resistant, and will subsequently survive changes in environmental conditions. This gene has an important role in the body's defense system, particularly against the activity of reactive oxygen species that can cause oxidative stress (Bowler *et al*., 1992; Gupta *et al*., 1993). The expression of SOD gene is incre -ased in soybean *Glycine max* which have drought (Hindarta, 2008).

MmCu/Zn-SOD gene plays a role in tolerance to aluminum stress in *N. benthamiana* and *N. tabacum* transgenic (Hannum, 2012); and successfully regenerated rice GMOs (Davis, 2012) and Jatropha (*J. curcas*) transgenic (Theresia, 2012). Based on these results, the research was conducted to determine the method of *MmCu/Zn-SOD* gene transformation into *K. alvarezii* to increase resistance to stress.

The method of gene transfer is DNA-mediated by *Agrobacterium tumefaciens* bacteria. *Agrobacterium* mediated gene transformation is widely reported in dicotyledenous crops such as tobacco

(Liu *et al*., 2004), potato, cucumber and tomato (Punja and Raharjo, 1996). However, there are limited studies about focusing on *Agrobacterium* mediated gene transformation on marine macroalgae such as seaweed. One such study introduced *Gus* and *GFP* genes with the 35S CaMV promoter using an *Agrobacterium* vector in *Porphyra yezoensis* which demonstrated gene expression in T1 and T2 generations (Cheney *et al.*, 2001).

The efficiency of transformation is affected by many factors such as *Agrobacterium* strain and concentration of *Agrobacterium* (OD), inoculation duration, co-cultivation time, pre-selection period, concentration of acetosyringone and other antibiotics in the media, type of explants and genotype (Wu *et al.*, 2006). Assembling of transgenic *K.alvarezii* has been done by introducing a citrate synthase gene (Daud *et al*., 2013; Handayani *et al.*, 2014) with a gene encoding lysozyme-C, (Fajriah *et al.*, (2015) with metallothionein gene type II, and with Kappa (K)-carrageenase gene (Rajamuddin *et al.*, 2016). However, the successful introduc-tion of genes was still low.

The objective of this study was to optimize a method for the *Agrobacterium* mediated *MmCu/Zn-SOD* gene transformation based on the optical density (OD), the duration of inoculation and co-cultivation period factors to increase the effectiveness of transgenesis in *K. alvarezii* and to evaluate the adaptability of seaweed *K. alvarezii MmCu/Zn-SOD*-transgenic against salinity stress.

2. MATERIALS AND METHODS

2.1. The seaweed and preparation of explants: The seaweed was derived from the Research Institute for Coastal Aquaculture, Maros-South Sulawesi and sterilized following the method described by Suryati and Mulyaningrum (2009).

2.2. Agrobacterium isolates: In this study, an *Agrobacterium* LBA 4404 isolates which carries pGWB5 expression vectors was used (3). At the T-DNA pGWB5, there was *neomycine phosphotransferase* II (npt II) and *green fluorescent protein* (gfp) genes with DNA fragment lengths od 456 bp with the 35S CAMV promoter (Fig. 1).

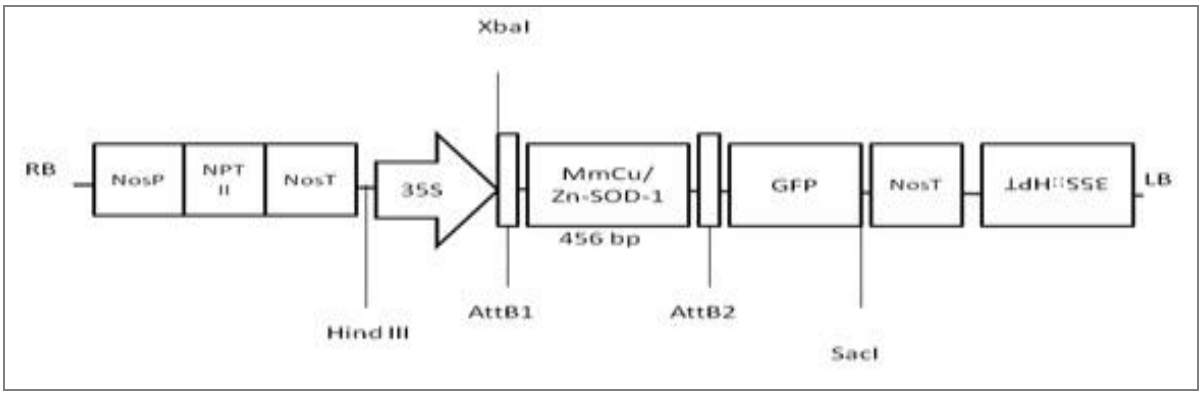


Figure- 1: The binary plasmids of pGWB5 that carry *MmCu/Zn-SOD* gene (pGWB-MmSOD) (Hannum 2012)

2.3. Bacterial culture: The *Agrobacterium* isolate which carries pGWB5 and contains the *MmCu/Zn-SOD* gene was propagated by growing single colony in Luria Broth liquid medium (LB) 10mL, containing 50µgmL^{-1}, kanamycin, 50µgmL^{-1} streptomycin and 50µgmL^{-1} hygromycin. It was shaken at 160rpm, 28°C for 18hours. Up to 1.5ml of bacteria was harvested and centrifuged at 5000 rpm for 5-10min at 28°C. The precipitate of *Agrobacterium* was resuspended using PES media and 0.1mM acetosyringone until reached OD (λ=600n M) of 0.4 and 0.5.

2.4. Co-cultivation, selection and regeneration of explants: *Agrobacterium* inoculation was completed by soaking seaweed explants in the *Agrobacterium* suspension (OD_{600} of 0.4 and 0.5) for 15, 20, 30 and 60min at 28ºC. After inoculation, the explants were drained on sterile filter paper and then co-cultivated by planting explants into solid co-cultivation media (28gL^{-1} seawater; 0.3% gelrite; 0.1mM acetosiringone) for 3 and 4days at 20-25°C in dark room. Per treatment, 10 explants with 3 replicates.

The explant was soaked for 10-15min in sea water media containing *cefotaxime* (100µgmL^{-1}) and then incubated in recovery media (solid PES 28gL^{-1} sterile seawater; 20mlL^{-1} PES, 0.4% bacto agar) for 7-10days. They were then cultured on selection media (PES solid 28gL^{-1} sea water; 20mlL^{-1} PES; 10mgL^{-1} hygromycin) for 14days. After selection, the explants were transferred to the recovery media for 14days, and then were maintained in liquid PES media (20mlL^{-1} PES and 28gL^{-1} sterile sea water) (Fig 2D).

2.5. Confirmation of transgenic explants by PCR analysis: The putative transgenic seaweed was confirmed by Polymerase Chain Reaction (PCR). Extraction of genomic DNA seaweed was modified by Edwards *et al.*, (1991) method. To confirm the incorporation of *MmCu/Zn-SOD* gene, we

used the specific primer pair: *MmSOD-F1*(5'-ATG GTGAAGGCTGTGGTTGT-3') and *MmSOD-R2* (5'-CATCTCCAACGGTGACATTG-3'). To confirm the incorporation of 35S CAMV promoter we used the specific primer pair: *35S-F2* (5'AAA CCTCCTCGGATTCCATT-3') and *MmSOD-R2* (CATCTCCTCCAACGGTGACATTG-3'). All of the specific primer pairs were constructed by Hannum (2012). In addition, PCR was also performed to amplify fragments of the 3'UTR seaweed actin as an internal control of DNA seaweed. The actin primers used for amplification were soybean actin primers. The PCR reaction was performed using 40cycles with pre-PCR conditions at 94°C for 15seconds, denaturation 94°C for 2min, annealing 55°C for 30sec; elongation of 72°C for 1 min and final elongation 72°C for 5min. The PCR products were then run on 1% agarose gel using TBE buffer. The DNA was visualized using a gel documentation system.

2.6. Salinity Stress Test: The shoot of explant transgenic and non-transgenic were used. They were cut into three pieces in the same length, and then were put into a container maintenance which already contain appropriate medium for salinity treatments. The salinity treatments for each explant of transgenic and non-GM consists of 15g/L (representing in low salinity), 30g/L (as a control) and 45g/L (representing in high salinity). Each treatment consists of three replicates. The observations were made on the challenge test of 7 and 14 days.

2.7. Data Analysis: Efficiency of regeneration was calculated from the number of explants that produced buds compared to the total number of explants. The number of explants which produced buds compared to the number of hygromycin-resistant explant was defined by the efficiency of putative bud. The efficiency of transformation was calculated from the number of plants carrying *MmCu/Zn-SOD* gene compared to the number of buds resistant (Cortina and Macia, 2004). Survival rate is a percentage of the number of live-sprouts compare to the total number of sprouts which have been challenge. Data were analyzed descriptively.

3. RESULTS

3.1. Survival rate of explants: In this study, the concentration of Agrobacterium by OD_{600} of 0.3-0.8 were tested. Explants which were only able to survive in OD_{600} of 0.4 and 0.5 (Table 1) were used, so that the bacterial density OD_{600} of 0.4 and 0.5 could be used to generate transgenic seaweed.

Experiments on OD_{600} of 0.4 and 0.5 with an inoculation duration of 30-60min and co-cultivation for 3-4days, resulted in a survival rate of 100% (Fig. 2A). Some explants grew some shoots on recovery media and had a survival rate of 100% too (Fig. 2B); some explants changed from green to pale and then died on hygromycin selection media, so a survival rate was 80% (Fig. 2C). In general, from a total of 480 explants that were introduced to all treatments, there were 198 hygromycin-resistant explants (41.25%), 118 explants that had budded (59.60%) and 9 transgenic explants (Fig 2E, Table 1). From 30 non-transgenic explants, 10 explants produced buds (Table 1). Regeneration efficiency of non-transgenic thallus (on non-selection media) was 33.33%, and thallus from non-transgenic on selection media entirely died (Fig. 2F).

Regenerated explants, after being selected by using hygromycine, can be seen in Fig 2D and 2E, the 2months of putative bud explants with a length of about 0.5cm (Fig 2D), and those of 4months old with a length of about 1-1.5cm (Fig 2D).

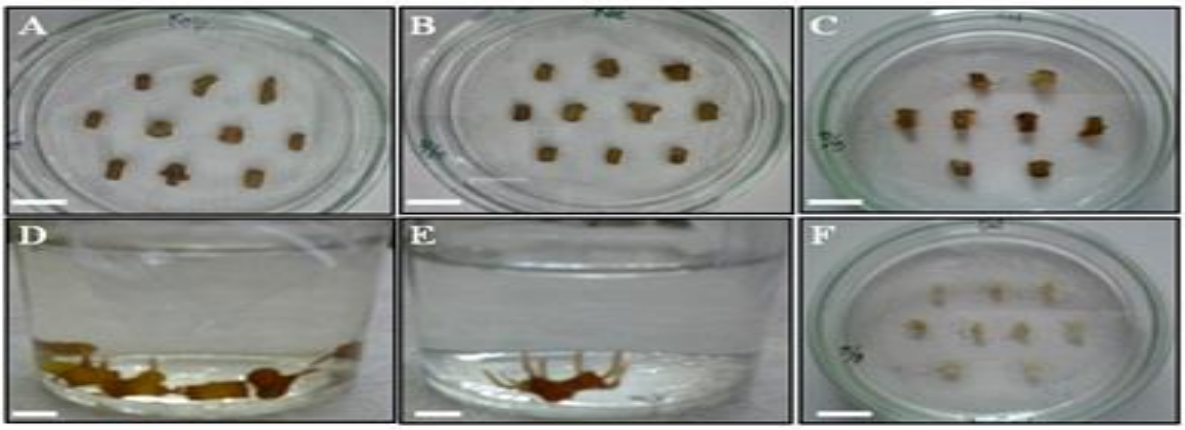


Figure- 2: Visual performance of the seaweed *K. alvarezii* [A = explants on the co-cultivation media (3-4days); B = explants on the recovery media (11-14days); C = explants on the selection media (24-28days); D = explants on the liquid media /liquid PES media (2months), E = transgenic explants on the PES media (4months); F = negative control (explants are all dead in the selection media), white color scale size = 1 cm].

Table 1. Results of the *MmSOD* gene transformation in the red seaweed *Kappaphycus alvarezii*

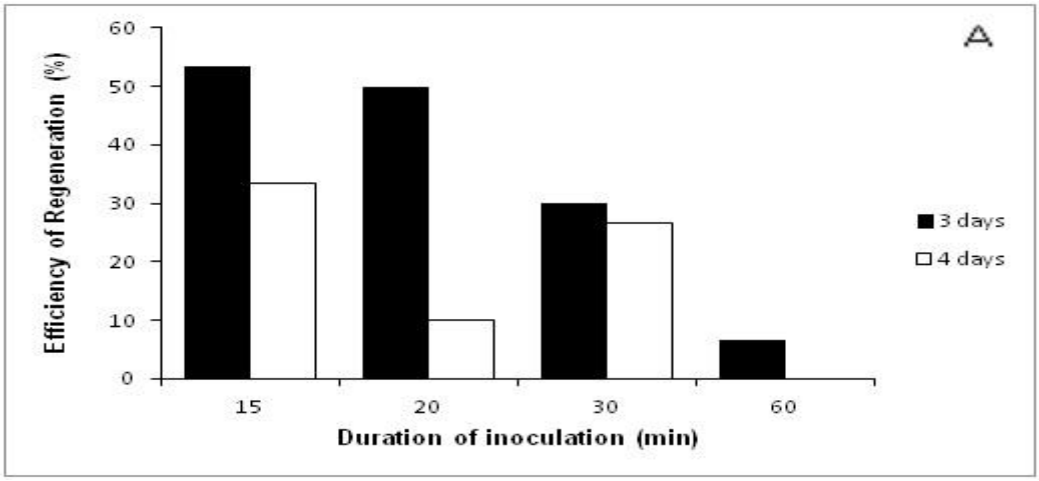
Experiments	OD_{600}	Inoculation duration (min)	Co-cultivation period (days)	Explant resistant hygromycin		Number of explant	
				Total	Percentage[a] (%)	Regene-ration	+ PCR
Trans-formation	0.40	15	3	24	80.00	16	0
			4	16	53.33	10	0
		20	3	22	73.33	15	0
			4	3	10.00	3	0
		30	3	15	50.00	9	1
			4	9	30.00	8	3
		60	3	4	13.33	2	1
			4	0	0.00	0	0
	0.50	15	3	22	73.33	10	0
			4	12	40.00	4	0
		20	3	21	70.00	10	0
			4	18	60.00	9	0
		30	3	20	66.67	14	1
			4	10	33.33	6	1
		60	3	2	6.67	2	2
			4	0	0.00	0	0
Total				**198**	**41.25**	**118**	**9**
Con [-1)]	-	-	-	0	0	0	0
Con [+2)]	-	-	-	-	-	10	-

a) Percentage of the resistant-hygomicin explant = The percentage of transformation = The number of hygromycin resistant explants / total number of explants X 100 %

1) non - transgenic explants were grown on hygromycin selection media (con = control)

2) non - transgenic explants were grown on PES media without hygromycin

3.2. Efficiency of regeneration: Generally at OD_{600} of 0.4, efficiency of regeneration (ER) decreases with increasing length of time of inoculation (Fig. 3A). The highest ER value (53,3%) was obtained from inoculation duration of 15min with co-cultivation of 3days, and the highest ER from co-cultivation of 4days was 33,33%. At the OD_{600} of 0.5, high regeneration efficiency was obtained from inoculation of 30min with co-cultivation period of 3days (46,67%), and duration of inoculation 15min with co-cultivation of 4days (30,33%) (Fig. 3B).



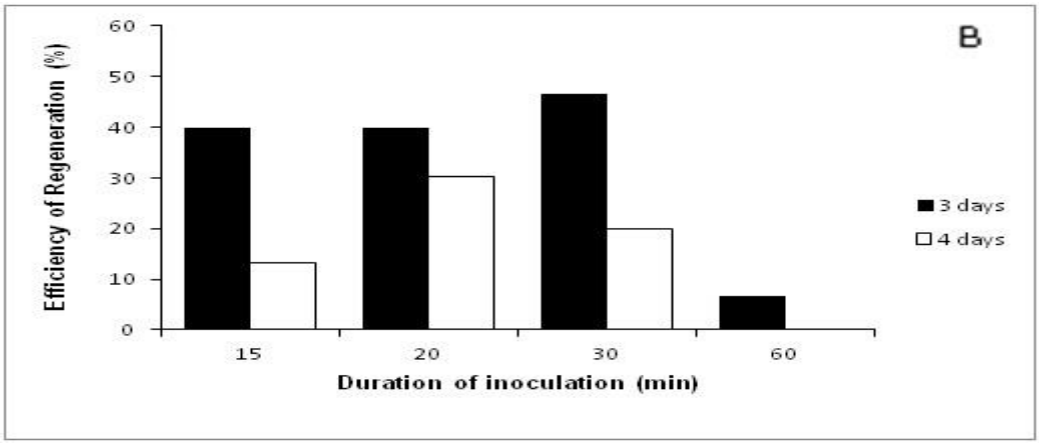


Figure -3: The efficiency of regeneration in 15, 20, 30 and 60min duration of inoculation with the co-cultivation periods of 3 and 4days at the OD_{600} of 0.4 (A) and 0.5 (B)

3.3. Efficiency of putative bud: Effect of treatment on the putative bud efficiency is presented in Fig. 4. The highest putative bud efficiency (100%) was obtained from OD_{600} of 0.4, inoculation duration of 20min with co-cultivation period of 4days treatment (Fig. 4A), and at OD_{600} of 0.5; 60min duration of inoculation and co-cultivation period of 3days treatment (Fig. 4B).

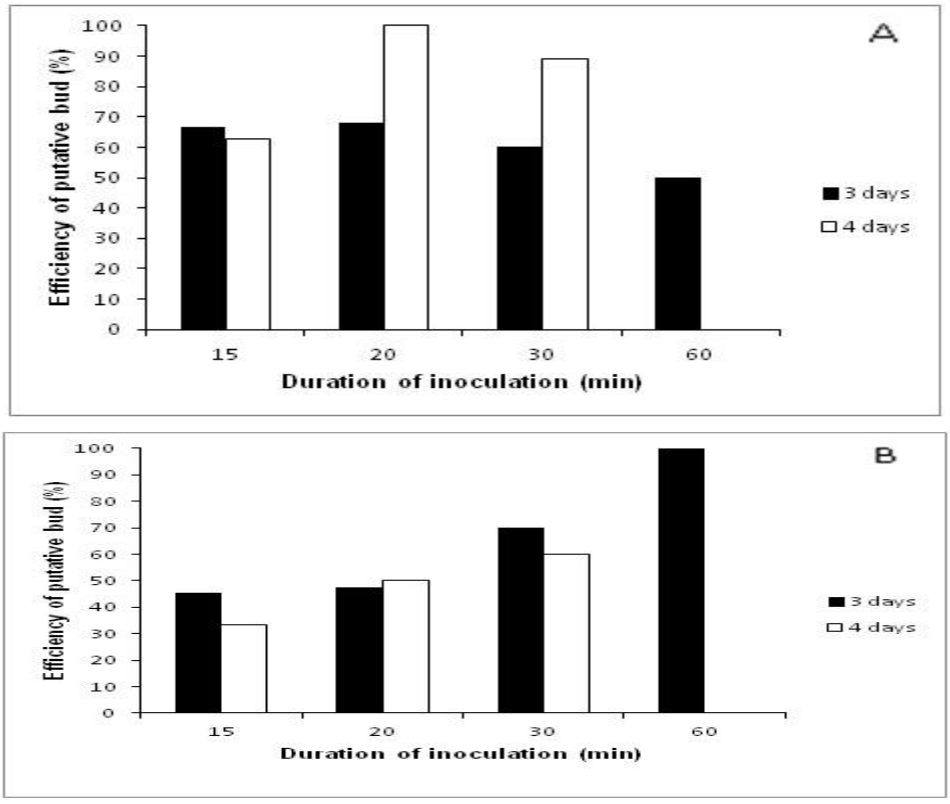


Figure -4: The efficiency of putative bud in 15, 20, 30 and 60min duration of inoculation with the co-cultivation periods of 3 and 4days at the OD_{600} of 0.4 (A) and 0.5 (B)

3.4. Efficiency of transformation: Effect of OD_{600}, duration of inoculation and co-cultivation period on the transformation efficiency (ET) is presented in Fig. 5. By using OD_{600} of 0.4 (Fig. 5A), the highest ET obtained was from inoculation duration of 30min with co-cultivation of 4days (33.3 %), followed by inoculation duration of 60min with 3days of co-cultivation (25%) and inoculation of 30min with 3days of co-cultivation treatment (6.67%). In OD_{600} of 0.5 (Fig. 5B), the highest ET was obtained from 60min inoculation duration with 3days of co-cultivation (100.0%). But, ET from inoculation duration of 30min, 4days of co-cultivation and OD_{600} of 0.5 was 10.0%, whereas 30min of inoculation and co-cultivation of 3days only gained 5% (Fig. 5B). Transgenic explants were not obtained through the other treatment combinations.

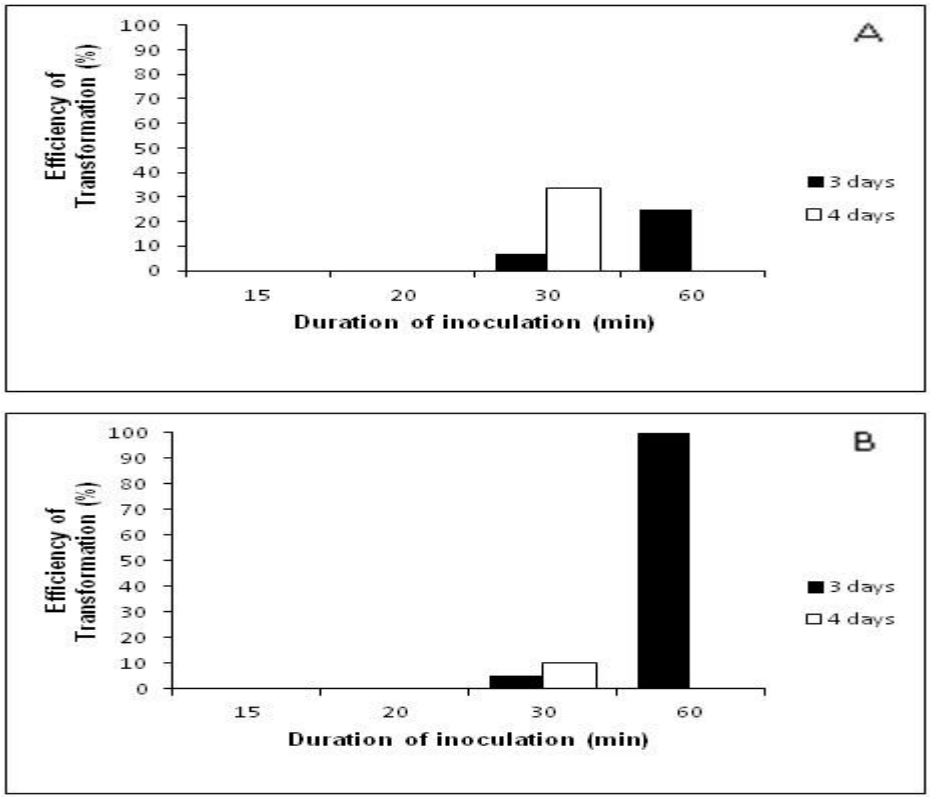


Figure 5. The efficiency of transformation in15, 20, 30 and 60min duration of inoculation with the co-cultivation periods of 3 and 4days at the OD_{600} of 0.4 (A) and 0.5 (B)

3.5. Identification of transgenic explants: Presence of *MmCu/Zn-SOD* gene was confirmed by PCR using three primer pairs (Fig. 6). PCR reaction with 35S CAMV promoter and *Mm Cu/ Zn-SOD* gene primers, showed amplification in each analysis transgenics, suggesting that the exogenous genes were successfully inserted into the genome. This was confirmed as the PCR results showed amplification of fragment in lanes T5, T6, T7, T8 and T9 as the transgenic, but no amplification of the non-transgenic seaweed in lane NT. All of the samples of transgenic explants (lanes T5, T6, T7, T8 and T9) gave the predicted DNA fragment of 633bp by *35SF-MmSODR2* primers (Fig. 6A) and of 456bp using *MmSODF-MmSOD-R2* primers (Fig. 6B) respectively. In addition, PCR using soybean actin primers as a DNA loading internal control of seaweed produced fragments with the size of 600bp in lanes NT , T5, T6, T7, T8 and T9 (Fig. 6C).

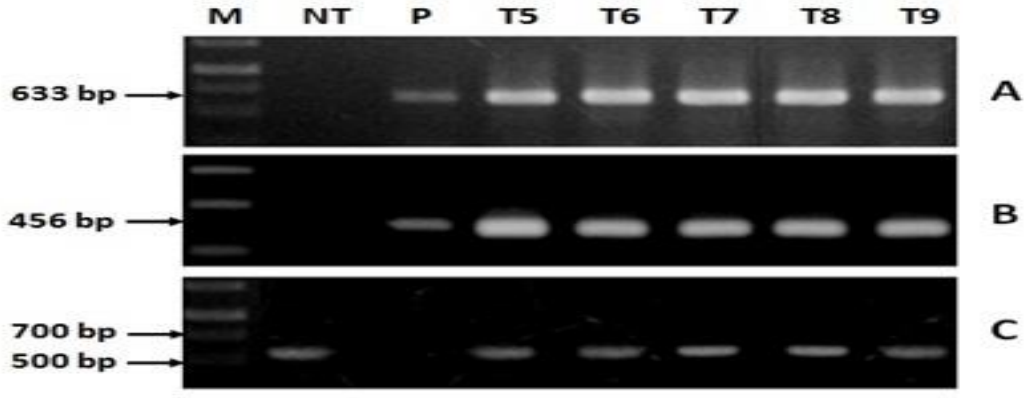


Figure -6: The results of DNA PCR analysis using 3 primers (A by using *35SF-MmSODR2* primers; B by using *MmSODF-MmSODR2* primers; and C by using soybean actin primers), M = marker 1kb DNA; NT is the negative control (non- transgenic seaweed); P is a positive control (plasmid pGWB5-MmSOD); T5-T9 are transgenic seaweeds).

3.6. Stress Salinity Test: The survival of explants during the test of salinity stress were shown in Figure 7. In salinity of 15g/L, the survival of non-transgenic (NT) and transgenic (T) explants were

33,33% and 100% respectively for the challenge test of 7days. On the 14th day of challenge test, all NT explants were killed, while T explants were still alive as much as 66.67%.

At salinity of 30g/L with the challenge test of 7days, the viability of sprout NT explant was 100%, as well as the T explant; with the 14days of challenge test, NT and T explants had survival rate of 100%. The survival of NT and T explants at salinity of 45g/L and 7days challenge test were 66.67% and 100% respectively. At the challenge test of 14days, all the NT explant death, whereas the survival rate of T explant was 100%.

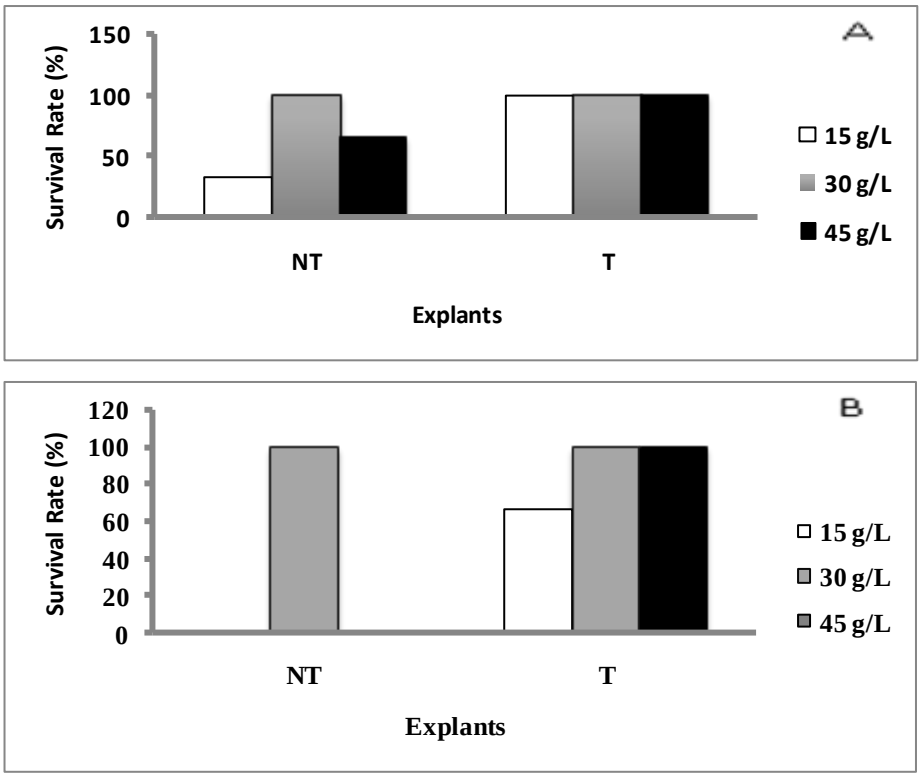


Figure 7. Survival rate (%) of *K. alvarezii MmCu/Zn-SOD*-transgenic at a different salinity with the challenge test of 7 days (A) and 14 days (B)

4. DISCUSSION

In preliminary studies, all explants died when using OD_{600} of less than 0.4 ($3,2x10^8 CFUmL^{-1}$) and above OD_{600} of 0.5 ($4x10^8 CFUmL^{-1}$). This may be due to bacterial overgrowth above OD_{600} of 0.5, as supported by Siregar, (1999) who found that using large bacterial populations decreases the number of surviving explants. The concentration of *A. tumefaciens* below OD_{600} of 0.4, was apparently not enough to infect explants seaweed. *Agrobacterium* as a tool of transformation requires certain conditions (specific density) in the optimal of gene transformation process (Gustian, 2002).

The study of SOD gene transformation has been done on Jatropa using OD_{600} of 0.4-0.5 (5), OD_{600} of 0.01 on Japonica rice (8) (Davis, 2012), and OD_{600} of 0.5-0.8 in *N. benthamiana* and *N. tabacum* (Hannum, 2012). Gene transformation in seaweed has been studied by incorporating different genes using OD_{600} of 0.5-1 (10, 11, 12). All of these studies generally showed that transgenic seaweed was produced using OD_{600} of 0.5 (Table 2).

Table 2. Results of the genetic transformation in the red seaweed *K. alvarezii* based on the multiple sources

OD_{600}	Inoculation duration (min)	Co-cultivation period (days)	Efficiency of Regenera-tion (%)	Efficiency of Putative bud (%)	Efficiency of Transforma-tion (%)	Sources
0,5	15	3	7,5	100	6,67	(10)
0,5-1	30	3	23,56	11,32	5,66	(11)
0,5-1	15-60	3	27,4	27,6	9,67	(12)
0,4	30	4	26,67	88,89	33,33	This study
0,5	60	3	6,67	100	100	This study

Our results are contrary to the earlier studies conducted by Paserang *et al.,* (2016), as they reported the highest efficiency with jatropa using OD_{600} of 0.1. The use of large bacterial populations in the gene transformation process needs to be considered as it relates to the growth phase of bacteria and vulnerability of explants at the inoculation duration and co-cultivation period (Siregar, 1999).

The efficiency of transformation was only found on the inoculation duration of 30 and 60min, both at OD_{600} of 0.4 and 0.5 with co-cultivation of 3days. However, it was not obtained in the 4days of co-cultivation and inoculation duration of 60min. These results indicate that *MmCu/Zn-SOD* gene was not inserted into the seaweed genome when inoculation duration was less than 30min, and only occurs at 30 and 60min. Unlike to the jatropa that produces the highest efficiency at inoculation duration of 20min (Kumar *et al.,* 2010). These differences are though t to be because cell walls of seaweed contain alginate, which plays an important role in maintenance of the tissue structure of algae (Rasyid, 2003), so it took longer for *Agrobacterium* to enter the cell.

Similar to the transformation efficiency, the efficiency of regeneration obtained in the inoculation duration of 30min (26.67%) was higher than reported by Handayani *et al.,* (2014), ie 23.56 % (in the same inoculation duration of 30 min) (Table 2). This is thought to be caused by the hygromycin concentrations used in this study which were lower ($10ugmL^{-1}$) than $20ugmL^{-1}$ (Handayani *et al.,* 2014). Lower doses of hygromycin supports the recovery of explants, demonstrated by an increase in the number of sprouting explants. In addition, the efficiency of putative bud on inoculation duration of 60min was 100%. This result, was also greater than reported by Handayani *et al.,* (2014) which was 11.32%, and Fajriah *et al.,* (2015) amounted to 27.60% (Table 2). This was presumably due to the length of inoculation duration (60min) longer than the ones used by Handayani *et al.,* (2014) and Fajriah *et al.,* (2015) ie 30min.

Co-cultivation period also affected the efficiency of regeneration, putative bud and transformation efficiency. The longer the time of co-cultivation, the lower the efficiency of regeneration. In this case, the efficiency of regeneration of 3days co-cultivation was greater than 4days in each treatment of inoculation duration. This is possibly because an *Agrobacterium* infection for a longer period of time would decrease the survival of explants (26) (Kuta and Tripathi, 2005). Our results are similar to the study conducted by Siregar, (1999), who reported that the short time of co-cultivation could increase the frequency of gene transformation.

At 3days co-cultivation, inoculation of 60min and OD_{600} of 0.5, obtained efficiency of regeneration, putative bud and transformation of 6,67%, 100% and 100% respectively, although the efficiency of regeneration was smaller than, the transformation and putative bud efficiency were larger than Fajriah *et al.,* (2015). They reported that regeneration efficiency, putative bud effi-ciency and transformation efficiency were 27.4%, 27.6% and 9.67% respectively. The low efficiency of regeneration obtained in this study compared to them, may be related to differences of co-cultivation media. In this study, solid co-cultivation media was used, while Fajriah *et al.,* (2015) used liquid co-cultivation media, which is used to imitate the natural habitat of the seaweed.

Regeneration efficiency and putative bud efficiency of the entire treatment were 24.58% and 59.60% respectively. Our results were greater than reported by Handayani *et al.,* (2014) with the regeneration efficiency of 23.56% and 11.32% of putative bud (Table 2). Differences in results may be due to differences in the strain and the location of seaweed used. Handayani *et al.,* (2014) used green seaweed cultivars originating from Lampung, while those used in this study were derived from the red cultivars Takalar, South Sulawesi. However, if the results were compared with (Fajriah *et al.,* 2015), the efficiency of regeneration obtained in this research was lower, but higher efficiency of putative bud was observed.

The overall success of the introduction of *MmCu/Zn-SOD* gene (measured by PCR analysis), resulted in 9 transgenic explants out of 198 hygromycin-resistant explants (4.54%). The low efficiency of transformation, putative bud efficiency and regeneration efficiency exhibited, suggest the method must be optimized so as to increase the successful introduction of *MmCu/Zn-SOD* gene in the Red seaweed *K. alvarezii*.

In terms of survival rate, the transgenic explants were able to live and adapt so they can survive both at the high and low salinity with survival rate of 100% in the challenge test of 7days, but at salinity of 15g/L with 14days challenge test, the survival rate of T explants becomes decreased (66.67%). In general, NT explants were not able to survive in the challenge test of 7days at salinity of 15g/L, so that its survival rate was only reached 33.33%, and even all of them were died on the 14-days challenge test. Similarly in salinity 45g/L, NT explants

survive only 66.67% in 7days challenge test, whereas all dead on the 14-days challenge test. This is supported by Hayashi et al. (2011) that seaweed *K. alvarezii* non-transgenic was dead after maintained of 3days at salinity of 15g/L. At salinity 30g/L, both transgenic and NT were all live (100%). At salinity of 45g/L, transgenic explants were survive of 100%. These results indicate that the introduction of *MmCu/Zn-SOD* gene was able to increase the durability of *K. alvarezii* to salinity stress. The same has been reported by Hannum (2012) and Darko et al., (2014) that gene expression and activity of antioxidant proteins were increase on the plants which tolerant to abiotic stress. Overexpression of SOD gene in various transgenic plants confers resistance to high salinity [Tanaka et al., 1999; Wang et al., (2010)]. Mortality of 100% occurs at salinity of 15 and 45g/L at 14-days challenge test, but on the seventh day challenge test the survival were 33.33% and 66.67% respectively. This indicates that NT was not tolerant in salinity 15g/L and 45g/L, while T explants were able to live and adapt to the low or high salinity. This is in contrast with the statement of Kaliaperumal et al., (2001) that all the wild-type algae tolerant at salinity 25-35g/L. Similar to Doty (1970) that seaweed *K. alvarezii* had optimum salinity range between 29-34g/L.

6. CONCLUSION

Kappaphycus alvarezii transgenic can be produced by *Agrobacterium tumefaciens* using OD_{600} of 0.4-0.5, 30-60min inoculation duration with co-cultivation period of 3-4days. The highest of putative bud efficiency, and transformation efficiency (100%) were obtained using OD_{600} of 0.5, inoculation duration of 60min, and 3days of co-cultivation.

Transgenic explants that carry encoding genes of *MmCu/Zn-SOD* tolerant to the salinity of 15g/L and salinity of 45g/L so all were survive, while all the non-transgenic were died.

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