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Full Length Article

Agrobacterium tumefaciens-Mediated Transformation of *MmCuZn-SOD* Gene to Sugarcane (*Saccharum officinarum* cv. PS864) for Acidic Soil Stress Tolerance

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Abstract

The main problems in the planting of sugarcane in acidic soil is the absence of cultivar of sugarcane that adaptive to the acid soil. Therefore, efforts to obtain cultivar of sugarcane that can adapt to environmental stress conditions acid are very important. The most effective and promising approach to obtain sugarcane with tolerant to acidic soils introducing the acidic soil tolerant gene into sugarcane using *Agrobacterium*-mediated transformation method. We have development of efficiency and regeneration from embryogenic callus and genetic transformation was established by using *MmCuZn-SOD* gene to acidic soil in sugarcane cv. PS864. The results showed that the highest percentage of survival callus embryogenic was obtain from OD_{600}0.2 of the *Agrobacterium* yielded 84.3%. Transformation efficiency was 33.3% that the highest number of transformation efficiency when embryogenic callus was cultured in 200 mg L^{-1} acetocyringone, with 10 min incubation time in bacterial suspension, 3 days for co-cultivated and selected on 250 mg L^{-1} cefotaxime. The presence of transgene was confirmed by PCR analysis showed that 10 planlets positive containing *MmCuZn-SOD* gene. The tolerance evaluation of transgenic and WT plants in selective medium containing of 500 mg L^{-1} aluminum and acidic soil showed the transgenic plants had well growth and survive in acidic soil with the highest of height and number of shoot compared with WT showed symptoms of wilting, necrosis and eventually die. The results obtained standard protocol of transformation *MmCuZn-SOD* gene in sugarcane cv. PS864 and transgenic plants had significantly higher tolerance to aluminum and acidic soil.

Keywords: Acidic soil; *Agrobacterium*; *MmCuZn-SOD*; Sugarcane; Transformation

Introduction

The expansion of planting area is one effort that can be done to increase sugarcane production but the directed expansion in to marginal lands, which have strong acidic soil is very difficult. The main limiting factor of sugarcane production in acidic soil is aluminum toxicity. Soil with pH of about 4, a toxic form of aluminum is dissolved into the soil solution, reducing of root growth and function as well as reductions in yields (Kochian *et al*., 2005; Ryan and Delhaize, 2010; Blancheteau *et al*., 2012). In abiotic stress such as aluminum, drought, heat and salinity stress in the cell can trigger Reactive Oxygen Species (ROS) product, inhibit respiration, deplete ATP and finally eliminated the ability of plants to grow (Vitorello *et al*., 2005; Dong *et al*., 2009). ROS such as hydroxyl radical (OH), superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), usually generated by normal cell metabolism, environmental stress and chemical that cause DNA strand breakage, membrane lipid peroxidation and enzymes inactivation (Krutika and Subramanian, 2007; Dong *et al*., 2009; Ueda *et al*., 2013). Organisms have evolved a complex system of antioxidant preparation mechanisms of non-enzymatic and enzymatic to scavenge ROS is to prevent oxidative stress (Gill and Tuteja, 2010; Ueda *et al*., 2013). One of the antioxidant enzymes, such as superoxide dismutases (SODs) is a group of metalloenzymes as a cell protection from superoxide radicals by catalyze dismutation the superoxide radical to molecular H_2O_2 and O_2. Several factors can affect the expression of SOD gene to abiotic stress such as drought in rice (Wang *et al*., 2005); in Arabidopsis (Xiao-fei *et al*., 2013), heat and salinity in *Trichoderma harzianum* (Yang *et al*., 2010), aluminum in ryegrass (Cartes *et al*., 2012) and acute ozone stress in rice (Ueda *et al*., 2013).

One strategy to overcome the problems is the use of acidic soil-tolerant varieties. But there is no current available sugarcane cultivar with tolerance toward acidic soil. The

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success of sugarcane varieties tolerant to stress acidic soil is determined by the availability of genes that control the tolerance. Genes that control and regulate stress tolerance acidic soil is not provided in the genomes of sugarcane (Gianotto *et al*., 2011) as the plant breeding efforts to improve stress tolerance to acidic soil using conventional approaches could not be done. Conventional genetic improvement in sugarcane takes longer time because of the reproductive system and have higher barriers for sugarcane that has a complex chromosome that causes variations in plant fertility rate (Gilbert *et al*., 2005; Mayavan *et al*., 2015).

Genetic engineering is a technique that is now widely used in plant breeding aimed at integrating genes into the genome of the target plant species relationships without any restrictions. Therefore, the approach of recombinant DNA technology to assemble transgenic plants through gene transformation technique using *Agrobacterium* is one other approach that can be done (Tzfiraz and Citovsky, 2006; Gurel *et al*., 2009; Shiromani *et al*., 2011; Zombori *et al*., 2011; Andrieu *et al*., 2012; Khan *et al*., 2013; Kumar *et al*., 2014; Mayavan *et al*., 2015; Islam *et al*., 2016; Li *et al*., 2016; Metwali *et al*., 2016; Shah *et al*., 2016; Wamiq *et al*., 2016). Transformation trough *Agrobacterium* offers more advantages than methods of direct transformation such as: the integration of a low copy numbers of genes into a plant chromosome, transfer a relatively large segment of DNA with a little rearrangement and being a simple in economical and efficient procedure.

MmCuZn-SOD gene is one of the genes has been reported to regulate and play a role in stress tolerance in which isolated from *Melastoma malabathricum* L. This gene was successfully constructed into a plasmid (Hannum, 2012). Analysis of integration and *MmCuZn-SOD* gene expression in sugarcane genome is very important because sugarcane is one species that has a complex chromosome. The success of the transformation of *MmCuZn-SOD* gene into the plant genome is determined by the accuracy of the selection of plant material for transformation, optimization transformation techniques the availability of a protocol for regeneration of transformants and use the appropriate analysis to detect strains carrying the gene targets transformant intact. The standard protocol genetic transformation which has been successfully applied to the types or varieties of certain crops will not always be directly applied to the types or varieties of other crops because the response of each species or even varieties may be different so that necessary modifications of existing techniques in order to obtain transformation techniques effective and efficient.

The transformation technique of *MmCuZn-SOD* gene through *Agrobacterium*-mediated method to improved tolerance to acidic soil has not been reported in transgenic sugarcane. Transformation of gene through *Agrobacterium* had major problem associated with necrosis or low of survival rate of the target cells (Sahoo *et al*., 2011). The cells that were inoculated Agrobacterium caused traumatic infection such as phenolization, oxidative burst (Wamiq *et al*., 2016) and subsequent cell death has been described as a frequent phenomenon with cell monocots (Shiromani *et al*., 2011; Andrieu *et al*., 2012). Genetically modified sugarcane is necessary to optimize a reproducible and efficient regeneration for successful transformation that will produce transgenic plants. Therefore, it was necessary to study to obtain a standard protocol of transformation *MmCuZn-SOD* gene in sugarcane throuh *Agrobacterium* with a success rate of transformation, integration and system-related stress tolerance of acidic soil to obtain putative of sugarcane tolerant to acidic soils.

Materials and Methods

Plant Material and Embryogenic Callus Induction

Embryogenic callus induction was performance in Indonesian Center for Agricultural Biotechnology & Genetic Resources (ICABIOGRAD), Bogor, Indonesia and Center for Estate Crops Research and Development (ICECRD), Bogor, Indonesia. PS864 sugarcane cultivar was used in this study. Explants were used for callus induction are the leaves that were leaf cylinders from sheath with three youngest sheets. Embryogenic callus as the target material were used for optimization and regeneration. The basal of the stem that constituted of the leaves sheath was sterilized as long as 30 min in 96% ethanol and then cultured on based MS (Murasshige and Skoog, 1962) containing with 3 mg L^{-1} 2,4-Dichlorophenoxyacetic acid (2,4-D); 100 mg L^{-1} glutamine; 2 g L^{-1} glycine and 500 mg L^{-1} casein hydrolyzate (CH) as optimization induction medium to induce somatic embryogenesis. Medium was solidified in 0.25% bacto-agar (w/v) with 5.8 of pH. The cultures were incubated in culture room at 25±1ºC under dim light condition. Embryogenic callus (compact, non-friable callus which develops root-like structures) was discarded after 14 days and only the selected embryogenic callus were used to transformation.

Agrobacterium Strains and Construct use for Transformation

The transformation method were performed in Laboratory of Biotechnology Research Indonesia–The Netherlands (BIORIN), Bogor Agricultural University, Bogor, Indonesia. The transformation using *MmCuZn-SOD* gene from *M. malabathricum* that was cloned into plasmid pGWB5 and inserted into *A. tumefaciens* LBA4404 (Hannum, 2012). Besides containing *MmCuZn-SOD* gene, this plasmid contains a selection marker genes *neomycine phosphotransferase II* (NPT II), green fluorescent protein (GFP) and *hygromycin phosphotransferase* (HPT). Pair of PCR primer: 35S-F (5'-AAACCTCCTCGATTCCATT-3') and MmSOD-R (TAACCCTGGAGACCAATGAT 5'-3') were used to detect integration of *MmCuZn-SOD* gene in transgenic sugarcane genome. This primer was designed based on the nucleotide sequences of CaMV 35S promoter

and *MmCuZn-SOD* gene (Fig. 1).

Preparation of Agrobacterium Culture

Agrobacterium strain LBA4404 as a primary culture was prepared from a freshly streaked plate by inoculating single colony in 20 mL of autoclaved liquid Luria Bertani (LB) medium supplemented with 50 mg L^{-1} hygromycin, 50 mg L^{-1} rifampicin, 50 mg L^{-1} streptomycin and 50 mg L^{-1} canamycin. The culture was incubatedas long as 24 h on a rotatory incubator shaker at 200 rpm in dark at 28ºC. The secondary culture was prepared in a 500 mL baffled flask containing100 mL LB medium (containing same antibiotics as used in the primary culture) by adding 0.4% of the primary culture and then grown under similar conditions. Once the OD_{600} reached~1.0 cells of *Agrobacterium* were pelleted by centrifugation at 8000×g for 15 min. Cells were resuspended in 20 mL of resuspension medium MS medium containing 200 mg L^{-1} acetosyringone to adjust OD_{600} the suspension of bacteria to 0.2 and 0.4.

Co-cultivation and Selection of Transformed Embryogenic Callus

Embryogenic callus was collected and Agro-infected by immersing them in the *Agrobacterium* LB4404 culture for 10, 20, 30 and 40 min with intermittent gentle shaking. The Agro-infected callus were dried on sterile filter paper and then transferred to the co-cultivation medium that was MS media supplemented with 3 mg L^{-1} 2,4-D; 2 g L^{-1} glycine; 100 mg L^{-1} glutamine and 500 mg L^{-1} CH and 200 mg L^{-1} acetosyringone and incubated at 25±1ºC in the dark for 3 and 6 days. Once slight growth of *Agrobacterium* appeared around most of the callus, the callus was rinsed 3–5 times with 100–250 mg L^{-1} cefotaxime in sterile distilled water and then dried on sterile filter paper and transferred into first selection medium as the same media at co-cultivation but containing 250 mg L^{-1} cefotaxime and 50 mg L^{-1} hygromycin and incubated for 12 days at 25±1ºC in dark. After the first selection, browning or black callus were discarded and only creamish healthy callus were shifted to the fresh media for second selection and maintained at 22±1ºC in dark. After second selection for 10 days, micro callus could be observed which were finally transferred to fresh media for third selection and allowed to proliferate for 5 days at 25±1ºC in dark condition.

Regeneration of Transformed Callus

Black or brown micro callus from third selection were discarded and only granular macro callus were cultured to MS media with 3 mg L^{-1} kinetin, 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime were added after autoclaving. Addition of kinetin in media culture was used to proliferation of somatic embyros structure scutellar and coleoptelar. These microcallus were incubated at 25±1ºC in light condition for 5 days for regeneration at the first phase. Regeneration in the second phase for development of shoots and roots these were transfered to fresh medium to regeneration with different type of media, i.e. MS media containing 0.5 mg L^{-1} IBA and 0.3 mg L^{-1} BAP as well as 30 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime and then incubated in light condition and sub cultured every 7 days.

Molecular Confirmation of Putative Transgenic Plants

Efficiency of transformation was calculated as the formula given below:

$$\text{Efficiency of transformation (\%)} = \frac{\text{Number of positive PCR}}{\text{Number of callus inoculated with } Agrobacterium} \text{x100}$$

Analysis *MmCuZn-SOD* gene integration in sugarcane genome were analyzed using polymerase chain reaction (PCR) analysis along with wild type (non-transgenic/WT). As much as 0.1-0.5 g of fress leaf tissue was used as a material for total genomic DNA isolation. Leaf tissue were treated with 50 mL NaOH solution and then incubated in boiling water for one minute and quickly transferred into the ice. Samples were neutralized by addition of 250 mM HCl and 50 mL of sample buffer, then sample boil for two minutes and immediately transferred to ice for two minutes. Samples were prepared for PCR analysis. Samples stored at a temperature of 4ºC was stable for two weeks. The principle of the method used to amplify DNA fragments isolated by PCR. Program thermal was set as follows: initial denaturation at a temperature of 95ºC for 5 min, denaturation at a temperature of 94ºC and annealing at 58ºC both of them for 30 sec and extension at temperature of 72ºC as long as 45 sec, the final extension 72ºC for 15 min. The volume of each reaction mixture containing 7.5 mL of 25 mL ddH2, 12.5 mL of solution master mix; P1 and P2 respectively 1.5 mL and 2 mL of template DNA. The reaction was run at 35 cycles of PCR machines. A total 5 µL PCR product is used for electrophoresis on a 1.0% agarose gel. Electrophoresis results were observed and photographed on a UV transiluminator.

Acidic Acid Tolerance Assay

Acidic soil was imposed on transgenic and WT plants tested in test tube with MS medium containing 500 mg L^{-1} aluminum as agent selection. The cultures were incubated in growth cabinet at 25±1°C under light condition for 60 days. Then evaluated the resilience of transgenic plants using acid soil in greenhouse for 30 days. The acid tolerance test was conducted using yellow podzolic acid from Jasinga, Bogor, Indonesia, which had pH 3.7. Observations were made daily after planting with observation parameters: number and height of shoot and number and length of roots.

Statistical Analysis

The experiments for transformation in this study were prepared based on a complete randomized design with three

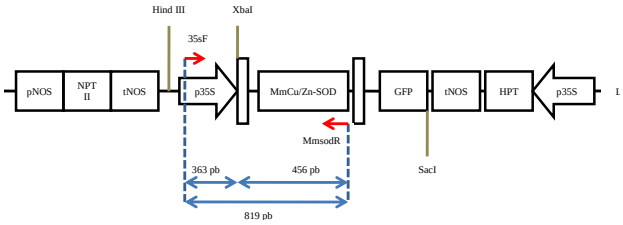

Fig. 1: Map of the physical on the T-DNA was flanked by a right border (RB) and left border (LB) on pGWB5 recombinant (Hannum, 2012). Red arrow indicate primers for amplification *MmCuZn-SOD* transgene in the genome of transgenic plants

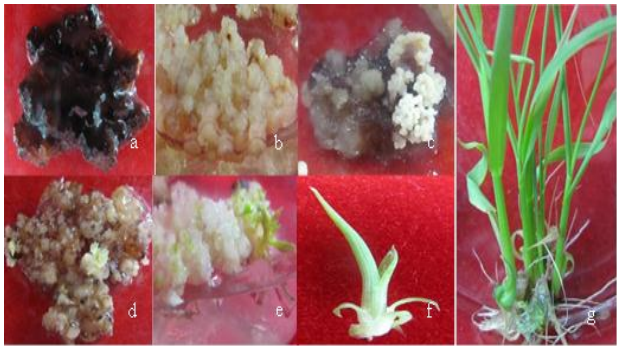

Fig. 2: Stages of transformation with *Agrobacterium* and regeneration of embryogenic callus of sugarcane. a) embryogenic callus without transformation in the selection medium containing hygromisin 50 mg L^{-1} as a positive control; b) infection of *Agrobacterium* and co-cultivation; c, d) selection 1 and 2 in the selection media containing hygromisin 50 mg L^{-1}; e, f) regeneration of shoots from micro callus in bright conditions; g) transgenic putative plants

replications in which each replication consisted of 10 calluses to optimize the frequency of sugar cane transformation regeneration. PS864. The data were recorded on the basis persent of callus survival in transformation treatment. The parameters for transformation efficiency were optimized in accordance with the expression of *MmCuZn-SOD* gene in shoots of the transfered sugarcane PS864. In treatment of acidic soil tolerance assay with aluminum as agent selection were prepared based on a complete randomized design with three-times manipulating 10 numbers planlets (number of positive PCR) and the data were recorded on the number of shoot, height of shoot, length and number of root after a periode of 60 days. Result were then analyzed using STAR Nebula, Statistical Tool for Agricultural Reseach (IRRI, 2013 Plant Breeding Genetics and Biotechnology Biometrics and Breeding Informatics). The means were compared by using Duncan's multiple range test at 5% level of significane (P<0.05). The results data were shown as mean±standart error (SE).

Results

Overview of the Method for Efficient Transformation and Regeneration

Embryogenic callus from non-embryogenic was essential that would affect the capability of transformation and regeneration from callus. Embryogenic callus were treated with Agro-infection through *Agrobacterium* that was carrying the T-DNA with *MmCuZn-SOD* gene (Fig. 1) and co-cultivated for 3 days (MS media supplemented with 3 mg L^{-1} 2,4-D; 100 mg L^{-1} glutamine; 2 g L^{-1} glycine; 500 mg L^{-1} CH and 200 mg L^{-1} acetosyringone). The growth of *Agrobacterium* could be visualized at the periphery of individual callus (Fig. 2b) these were displace to selection medium as the same as co-cultivation medium with added 50 mg L^{-1} hygromycin and 250 mg L^{-1} cefotaxime. The 12 days after culture, WT callus turned brownish while other remained creamy (Fig. 2c). Callus with creamy colour were shifted to fresh medium for cycle selection where small micro callus started growing on the mother callus (Fig. 2d). Micro callus were separated from the mother callus and shifted to fresh medium for the third selection (Fig. 2e). This step indicate that the proliferation of micro callus was transfered to first regeneration medium and maintained as long as 7 days in the dark condition. In this step, generally of micro callus developed into somatic embryos (Fig. 2f). These were transfered to the second medium of regeneration and cultured for 7 days in light condition and emerge green-spots which later developed into shoots (Fig. 2g).

Identification of transgenic in the present study, two different levels of OD_{600} i.e., 0.2 and 0.4 were applied for the best range of experimental infection, hygromycin selection and subsequently on transformation efficiency of sugarcane. The results (Fig. 3) showed that optimum OD_{600}0.2 used for callus embryogenic infection yielded 84.3% survival frequency while on other hand the higher of OD, more than 20 min in Agro-infection and co-cultivation more for 3 days was minimum survival frequency.

An incubation time of 10 min and inoculum density of 0.2 and 0.4 (OD_{600}) of the *Agrobacterium* increase the transformation eficiency of explans by 33.3 and 13.3% respectively (Fig. 4). In addition, overgrowth and bacterial leaching on explant occurred in >20 min *Agrobacterium* incubation condition and 6 days for co-cultivation.

Molecular Analysis of Transgene Integration *MmCuZn-SOD*

Identification of transgenic plants was performed by detecting the presence of *MmCuZn-SOD* transgenes in transformed plants using PCR (Fig. 6). Primers were used the 35S-F and *MmCuZn-SOD*-R2 with the size of the DNA fragments of 633 base pairs (bp) derived from the amplification section 3 'end of the 35S CaMV promoter (363 bp) and part of the 3' end coding region of *MmCu/Zn-SOD* gene (270 bp).

Acidic Acid Tolerance Assay

Aluminum-tolerance assay results showed that transgenic plant were survive after 60 days in medium selection containing 500 mg L^{-1} aluminum as agent selection, remain green and healthy at extreme concentration of aluminum while all WT showed browning and then death. These results suggested that there was significant different between transgenic and WT growth in aluminum stress conditions (Fig. 7). Transgenic plants were tested in acidic soil after treatment in selection medium with 500 mg L^{-1} aluminum. Transgenic plants showed well growth and survive in acidic soil with the highest of height and number of shoot compared with WT in normal soil (Fig. 7).

Discussion

The success of genetic transformation system or introducing the target gene into sugarcane cell was affected by a number of distinct factors, such as: OD of *Agrobacterium*, acetosyringone concentration, co-cultivation period, concentration of different antibiotics and different explants sources (Briza *et al*., 2008; Cho *et al*., 2008; Zong *et al*., 2010; Zombori *et al*., 2011; Khan *et al*., 2013; Kumar *et al*., 2014; Li *et al*., 2016). All these factors affect the value of transformation efficiency. Optimum OD of *Agrobacterium* to infect is pre-requisite, high OD *Agrobacterium* was resulted browning embryogenic callus, lower cell survival and recovery, overgrowth and low transformation efficiency that leads death of desired embryogenic callus. Zong *et al*. (2010); Kumar *et al*. (2014) has been reported that the highest transformation efficiency causing death of explants when OD_{600} more than 0.6 due to an excessive growth of bacteria or overgrowth. Co-cultivation period also affects the success of transformation activities. Normal co-cultivation time increase transformation efficiency and longer co-cultivation period subsequenlty result overgrowth of *Agrobacterium* and subsequent death of desired explans. Kumar *et al*. (2014) obtained that maximum incubation time in *Agrobacterium* suspension was 20 min but easy explants sprout on 10 min incubation time edge only slightly resistant to hygromycin antibiotic. The opposite result was reported by Zong *et al*. (2010) that incubation time in *Agrobacterium* was not significantly different between 10, 20 and 30 min. However, the recommended time to obtain persentage of survival calli was incubation in 0.2 OD of *Agrobacterium* for 10 min and 3 days of co-cultivation (Fig. 3). Longer duration of incubation in *Agrobacterium* could be attributed to excessive injury and loss of outermost (peripheral) cell that might have greater chance of transformation because of the directly contact with *Agrobacterium*.

Manickavasagam *et al*. (2009) observated that co-cultivation periode of more than 5 days leads to suppression of shoot emergence and low of transformation frequency. The variation result with the other research might be due to changes in explant used, infection and co-cultivation period

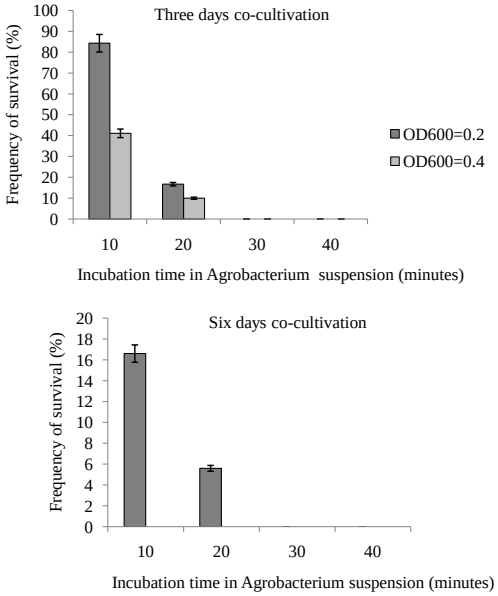

Fig. 3: Effect incubation time of *Agrobacterium* strain LBA4404 suspension with optical density dan periode of co-cultivation to frequency of survival (%). Values represent means±SE at P<0.05 probability level

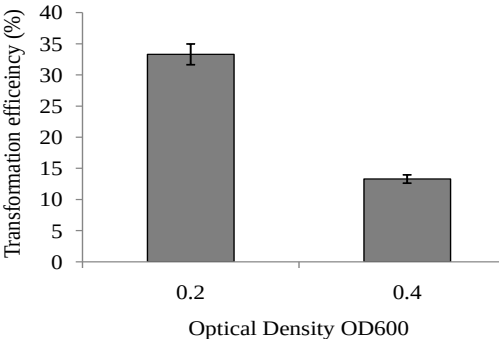

Fig. 4: Effect incubation time of *Agrobacterium* strain LBA4404 suspension for 10 min with optical density and co-cultivationfor 3 days to transformation efficiency (%). Values represent means±SE at P<0.05 probability level

and strain as well as the construct used. The data demonstrated the OD, incubation time in *Agrobacterium* suspension, periode of co-cultivation could enhance the interaction between *Agrobcaterium* and the explans cells. It might be due to the fact that T-DNA was governed by virulence genes and influenced by wounded plant cells (Wamiq *et al*., 2016).

Several realease compound attact the bacterium to wounded plant cell, following co-cultivation of *Agrobacterium* in media containing signal molecule, such as acetocyringone that plays an important role to enhance the transformation efficiency. The transformation efficiency was 33.33% (Fig. 4) that showed the highest transformation

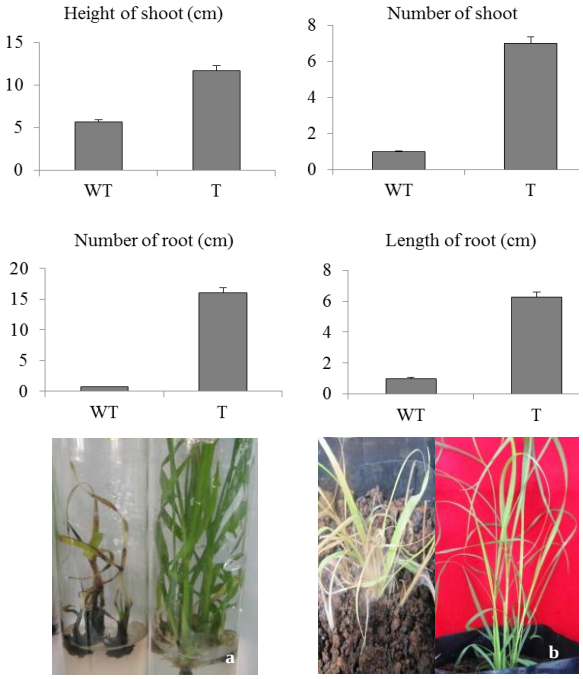

Fig. 7: Effect aluminum treatment on transgenic and non transgenic (WT) plants to height and number of shoot and length and number of root.Values represent means±SE at P<0.05 probability level were calculated from results of three independent experiment with 10 replications. a) Performance of WT (left) and transgenic (right) under 500 mg L^{-1} aluminum stress after 60 days of culture; b) Performance WT (left) and transgenic (right) in acidic soil after 30 days of planting

efficiency compared with research result by Mayavan *et al*. (2015) in sugarcane varieties Co 62175 (29.6%) and Co 6907 (32.6%); Wamiq *et al*. (2016) in cotton (20%).

The process of embryogenic callus growth into plantlets using hygromycin and cefotaxime that was a part of the composition of the media. Cefotaxime produced tremendous effect of controlling *Agrobacterium* overgrowth and thus produce maximum number of shoots regeneration. The addition of cefotaxime in the media contributed to kill the *Agrobacterium* that still contained in the explants. The function of hygromycin antibiotic in the media would select explants transformants because it served as the selection agent that would kill cells that did not express the gene *hpt*. The addition of hygromycin in the media caused morphological change on explants result, some began to experience browning, necrosis or even death. The *hpt* gene, the selectable marker in pGWB-*MmCuZn-SOD* encoding *hygromycin phosphoransferase* and confers resistance to hygromycin. The positive embryonic callus were resistance to selection medium containing hygromycin antibiotic caused by effect of expression of *hpt* geneproduce *hygromycin phosphotransferase*, which will inactivate hygromycin antibiotic and affect to the plants become resistant to

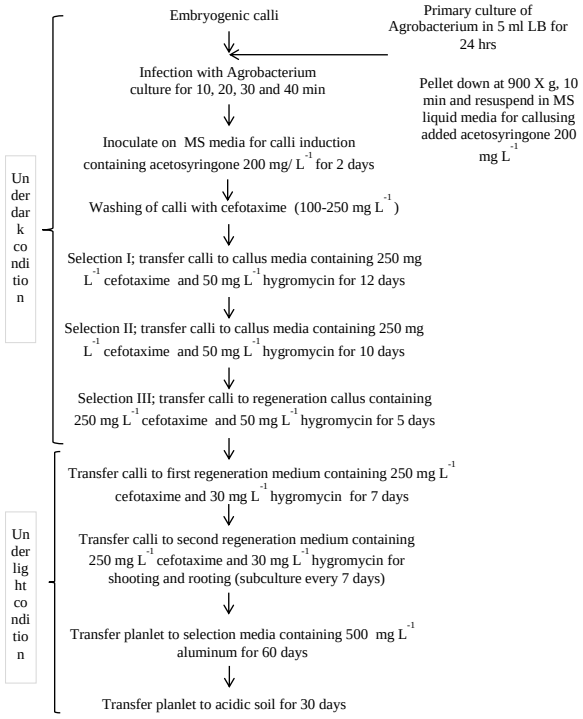

Fig. 5: Flow diagram of the stages of transformation *MmCuZn-SOD* gene using vectors *A. tumafaciens* LB4404

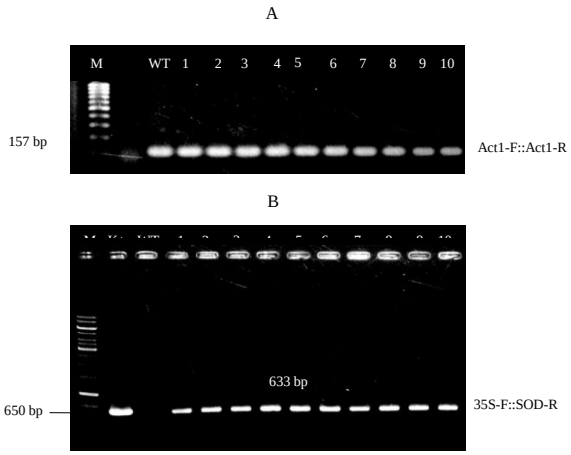

Fig. 6: Moleculer analysis of *SOD* gene by PCR. A. The amplified product from actin primer in rice (OsAct1); M=100 bp DNA ladder (Act1F::Act1-R); B. The amplified product from primer 35S forward (sekuen promoter 35S CaMV) and primer SOD reverse; M=1 kb DNA ladder (35S-F::SOD-R); K+=plasmid control (pGWB5-MmCU/Zn-SOD); WT=non transgenik; 1-10=positive transgenic

hygromycin and grow well in selection medium-containing antibiotic. The flow chart of the current study to optimize transformation *MmCuZn-SOD* in sugarcane was summerized in Fig. 5.

The presence of transgene was confirmed by moleculer analysis of the transformed planlets carrying *MmCuZn-SOD* by PCR technique (Fig. 6). However, no amplification fragment was observed in DNA extracted from WT shoots.

To futher investigate how *MmCuZn-SOD* functions in stress signaling, we compared the growth of WT and transgenic plants under aluminum stress conditions and acidic soil. It was apperent that under normal condition, the transgenic plants displayed similar morphological phenotypes as that of WT. After 60 days exposure to aluminum conditions all the WT death (Fig. 7a). These result indicated that transgenic plants apparent over-expression under aluminum stress condition due to have a foreign gene against abiotic stress significantly increased the performance of plants (Fig. 7). A correlation exists between oxidative stress and aluminum phytotoxicity. The genes of oxidative stress induced by aluminum including those encoding e.g. SOD. These genes were directly or indirectly play a role in signaling events in many organisms (Xiao-fei *et al*., 2013) and were proposed to be involved in a signaling system that was associated with aluminum stress (Blancheteau *et al*., 2012). Therefore, it has been suggested that there might be a general induction or signaling mechanism for response to aluminum toxicity and oxidative stress. After treatment in aluminum, transgenic plants were tested in acidic soil. Transgenic plants showed survival and well growth in acidic soil with the highest of height and number of shoot compared with WT in normal soil (Fig. 7b). Blancheteau *et al*. (2012) reported that ROS induced stress may be due to the acidic soil (low pH) stress than aluminum.

The tolerance of transgenic plants to aluminum and acidic soil that coenzyme defense system by deleting superoxide anion. Difficulties of environmental often to increase in decreased oxygen species generation and then SOD has been exteded to be important in stress tolerance. Many researches declared that stress of oxidative was an affecting component of environmental stress. SOD overproduction was found to be connecting with an increased tolerance to oxidative stress and found that SOD plays an important role in the protection such as *Trichoderma harzianum* to heat and salt stress, sugarcane to salinity (Kumar *et al*., 2014) and Arabidopsis to drougt (Xiao-fei *et al*., 2013).

Conclusion

In conclusion, transformation efficiency of *MmCuZn-SOD* gene on embyogenesis callus of sugarcane was established by using 0.2 OD_{600} of *Agrobacterium*, 250 mg L^{-1} acetocyringone, incubation time in suspension of *Agrobacterium* for 10 min and 3 days co-cultivated. Transgenic plants that were tolerance to acid soil obtained by testing transgenic plants for aluminum and acidic soil including WT, which showed that only transgenic plants tolerant to acid soil, while the WT showed symptoms of wilting, necrosis and eventually die. Transgenic plants developed in this study could be used further to support plant breeding programs to promote sugarcane acidic soil tolerant cultivars.

Aknowlegdements

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