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Stable Transformation of Gene by Direct Practical Bombardment Method in *Leucaena leucocephala*

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ABSTRACT

The embryo of Leucaena leucocephala was culture on MS medium after transformation by direct partical bombardment method. The shoot regeneration was achieved on full strength MS medium supplemented with 6 mgl^{-1} BAP and 1 mgl^{-1} NAA while rooting was induced in a half strength MS medium containing 3 mgl^{-1} IBA and 0.05 mgl^{-1} kinetin. The transformants were selected on shoot regeneration medium containing 100 µg ml kanamycin. The evidence for expression of the gus gene was established by histochemical and fluorognic gus assay of stem and leaf sections of these transformants. The analysis of transformants by southern hybridization confirmed the interaction of gus gene into the plant genome.

Keywords: *Leucaena leucocephala, Gene, Gene gun method, Transformants, Regenerations.*

INTRODUCTION

Leucaena leucocephala is a versatile fast growing and widely planted leguminous tree It is a potential source of firewood, industrial fuel, organic fertilizer, timber and raw material for pulp and paper industry (Yietmeyer & Cottom, 1977). In recent years, there has been increasing interest in commercializing in *vitro* propagation and genetic transformation of *Leucaena leucocephala* for paper industry. A high degree of lignifications is a formidable hurdle in the optimal utilization of plant biomass for pulp and paper industry. The paper manufacture involves separation of lignin from cellulose *via* expensive energy and chemical internship and aften polluting processes to acquire better quality of paper (Dwivedi et al., 1994 & Pilate et al., 2002) The biotechnologically modification of this attractive perspective for obtaining plants with improved qualities. Thus, genetically engineered *Leucaena leucocephala* producing modified lignin will lead to enhanced the contribution of these plants for paper and pulp industry, The technique for genetic transformation of this legume tree from the embryo was yet not established This paper described gene gun method protocol for *Leucaena leucocephala* through direct embryo culture use for shoot and root regeneration.

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MATERIALS AND METHODS

Plant material

The seeds of the *Leucaena leucocephala* were surface sterilized and placed it into a sterilized petri plate which contain sterile water for 24 hrs at 28° C. Then the embryo were removed from the seed and allowed to germinate on the full MS medium for 24 hours (Murashige & Skoog, 1962) The embryo of *Leucaena Leucocephala* was place into the osmotic tissue culture medium for 12 hours prior to shooting.

Gene gun method

Take 25 mg tungsten in microcentrifuge tube and add 250 µl absolute ethanol, vortex and spine for four time. Remove the alcohol carefully and resuspend the tungsten in sterile water. Vortex this solution and spine again then add 50 µl fresh sterile water. The 10 µl plasmid DNA as *Pseudomonas cambia* vector containing gus gene and kanamycin resistant gene was added into aliquot of tungsten and vertex then 50 µl of 2.5 M $CaCl_2$ and 20 µl of 100 mM freshly prepared spermdine was added and vortex, place this mixture on ice for 5 minutes. The supernatant remove and place 30 µl tungsten coated DNA back on ice for shooting. Take 5 µl of coated DNA for every shots. The embryo was placed on sterile petri dish and place it in the center of sliding tray and the baffle over the top. The sliding tray was 10 cm distance of the target shelf and the 600 mmhg-1 vacuum was adjusted into the chamber and the helium gas was allow to flow into solenoid. The DNA coated tungsten particles accelerated with the gas stream at the time of shooting.

Plant regeneration

The shoot regeneration medium comprised full strength of MS medium with 6 mgl^{-1} BAP and 1 mgl^{-1} NAA. The positive transform selected on the shoot regeneration medium supplemented with 100 µg ml^{-1} kanamycin. The root induction was achieved on half strength of MS medium with 3 mgl^{-1} IBA and 0.05 mgl^{-1} kinetin The regenerated plantlets were transplanted to potting soil and then maintained in a culture room for 7 days before transfer to green house where they were grow to maturity.

Histochemical gus assay

The tissue is incubated in reaction buffer containing 2 mM 5-bromo -4- chlro -3- indolyl glucuronide (X-glu), 10 µl ml^{-1} NN-dimethyl formamide buffer, 50 mM sodium phosphate buffer (pH-7.0), 0.1% Triton X-100, 10 mM EDTA, 0.5 mM potassium ferrocynide. Tissue is infiltered under vacuum till bubbles were come out and incubated at 37°C for overnight and screened for blue staining indicating gus gene activity under a microscope (Jefferson, 1987).

Fluorogenic gus assay

Tissue was grind in liquid nitrogen to fine power and add 0.1mg μl^{-1} gus assay buffer containing 50 mM sodium phosphate buffer (pH-7.0) 10 mM β – mercaptoethanal, 10 mM EDTA, 0.1% SDS, 0.1% Triton x-100 and centrifuge at 12,000 rpm for 15 min. Take 90 µl supernatant and put on ice then add 10 µl 4-methyl umbelliferyle D-glucaronide (10 X-MUG). Incubate at 10 min, add methanol to final concentration of 20% and once again incubate at 37°C for 24 hrs. The 900 µl (0.2 M) sodium carbonate added to stop the reaction. The intensity of fluorescence was visualized under UV light.

Southern blot analysis

Total genomic DNA was isolated from wild type and transformed plant tissue by the method Davis et al 1980. Twenty µg DNA of each sample was digested with Hind -III and subjected to electrophoresis on 1% agarose gel and transferred to nylon membrane. The probe DNA was labeled with (α-32P) dCTP using a random primer labeling kit. The pre hybridization and hybridization was performed in 6 X saline sodium citrate (SSC), 5 X Denhardts solution and 0.5% SDS at 65°C for overnight. The membrane was finally washed with 0.2 X SSC and 0.1 % SDS at 65°C for 30 min and autoradiograph on Kodak Biomax MS film (Sambrook et al., 1989).

RESULTS AND DISCUSSION

Plant transformation

The embryos were directly used for the transformation of *Leucaena leucocephala*. The transforments were selected on kanamycin containing shoot regeneration medium. After

three weeks of cultivation, shoots were produced directly from the embryo without any intermediate callus phase (Fig.1). Then these shoots were transfer to root induction medium. The root initiation was observed within 15 days of culture. Rooted plantlets were gradually acclimatized to soil condition for one week inside the culture room. The plantlets were then transferred to soil in green house where they successfully adapted for natural environment conditions.

The plant regeneration system was developed before genetic transformation of *Leucaena leucocephala*. The nodal explants form mature trees and seeding raised cotyledonary node explants were used as for the transformation by using *Agrobacterium tumefaciens*. These explants have problem for regeneration of nature plants. The phenols were leaching into the medium from the cuts ends the explants. Another problem callusing were observed in the transforments where as no callusing in non transformants plants.

We used embryo of *Leucaenal leucocephala* for the genetic transformation by using direct particle bombardment method. The transformation efficiency was found to 80-85% based on the transient embryo survives after selection on the 100 µg kanamycin containing medium. The different concentration of kanamycin was used for selecting of transforment. The lowest concentration 9 mg l^{-1} kanamycin was used by Mullins et al. (1997) for the tree species.

The highest concentration of 200 mgl^{-1} kanamycin was used by Cheng et al. (1996) and Sharma and Anajaih (2002) for the selection of transformants in case of grain legumes.

The high transformation efficiency was reported about 24% from the stem segments of the true legume *Robinia pseudoacacia* (Igasaki et al., 2000). The lowest transformation efficiency was reported 2% from most of the legume (Kar et al., 1996, Yan et al., 2000, Krishanmuthy et al., 2000 & Jaiwal et al., 2001).

Histochemical assay

Histochemical gus localization pattern is develop from stem sections as blue spots in the transformants revealed the expression of the gus gene (Fig.2). The non transformants did not stain positive blue colour with X-glue substrate.

The intensity of fluorescence in the fluorogenic gus assay was increased gradually with increasing incubation period for transformed tissue suggesting the increasing activity of the gus enzymes, where as non transformants showed no signs of florescence (Fig.3)

Southern blots

The presence of gus gene in the transformants were analyzed by southern blots hybridization. The figure four showed a strong 2.8 kb DNA hybridization band in lane 2. The gus probe did not hybridize to the genomic DNA extracted form non transformed plants as lane-1.

The result of southern blot hybridization revealed a strong hybridization band of 2.8 Kb in case of transformants only. This indicated the stable integration of gus gene into the genome of *Leucaena leucocephala*. Thus, a protocol of genetic transformation directly from the embryo has been developed. This report produce transgenic *Leucaena leucocephala* for better efficiency an improved quality, such as reduced lignin, mimosine contents and resistance to insect.

Fig. 1: The seeds upper and swollen embryo lower of *Leucaena leucocephala* plant

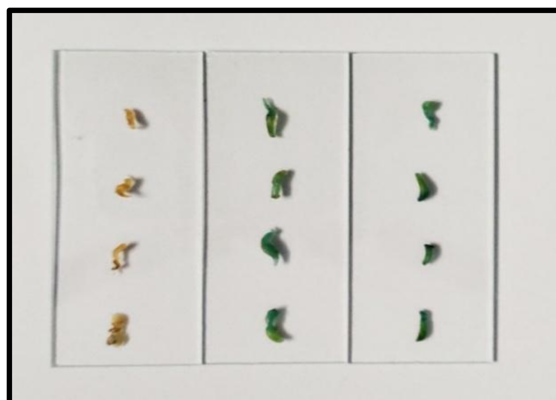

Fig. 2: The histochemical gus assay of stem
1-non transformant plant 2 and 3- transformant plant

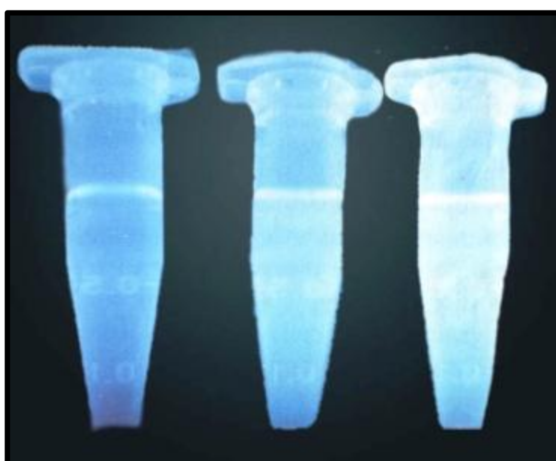

Fig. 3: The fluorogenic gus assay of leaves form transformant plant
The blue color indicating expression of the gus gene.1-non transformant plant 2 and 3-transformant plant at 12 hours and 24 hours of incubation period respectively.

Fig. 4: Southern blot hybridization analysis.
Lane-1-shown as non transformant plant.
Lane-2 and 3 represented 2.8 kb DNA fragment in transformant plants

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