

Development of an *Agrobacterium*-mediated transformation protocol for the tree-legume *Leucaena leucocephala* using immature zygotic embryos

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Abstract The tree-legume *Leucaena leucocephala* (leucaena) is used as a perennial fodder because of its fast-growing foliage, which is high in protein content. The use of leucaena as a fodder is however restricted due to the presence of the toxin mimosine. Improvements in the nutritional contents as well as other agronomic traits of leucaena can be accomplished through genetic transformation. The objective of this research was to develop a transformation protocol for leucaena using phosphinothricin resistance as the plant selectable marker. Explants obtained from immature zygotic embryos infected with the *Agrobacterium tumefaciens* strain C58C1 containing the binary plasmid pCAMBIA3201 produced four putative transformed leucaena plants. Transformation was confirmed by PCR, RT-PCR, Southern blot, Western analyses, GUS-specific enzyme activity and herbicide leaf spraying assay. A transformation efficiency of 2% was established using this protocol.

Keywords *Agrobacterium tumefaciens* · Genetic engineering · Herbicide-resistance · Leguminous trees · Leucaena · Transgenic woody plants · Immature zygotic embryos

Abbreviations

2,4-D 2,4-Dichlorophenoxyacetic acid
BA Benzyladenine
C/S/RIM Callus/shoot/root induction medium
GUS β -Glucuronidase

IBA Indole butyric acid
NAA Naphthalene acetic acid

Introduction

Leucaena leucocephala de Wit. (leucaena) belongs to the family *Fabaceae*, subfamily *Mimosoideae*, and is the best known species of the genus *Leucaena*. Leucaena is a fast-growing, nitrogen-fixing leguminous tree that is grown in many tropical and subtropical countries. It can grow under different soil and environmental conditions, such as alkaline and arid regions, and it is highly resistant to insects and pathogens. Because of its deep root system and ability to fix nitrogen, it is considered to be an ideal plant to control soil erosion and to enhance fertility in nitrogen-depleted soils. In many countries, leucaena is used as a fuelwood, and also for producing pulp and charcoal. Leucaena foliage is especially useful as a fodder for livestock animals because of its high nutritional values (Jones 1979). The protein content of leucaena is much higher (15–18%) compared to tropical grasses and cereal straws (3–5%), making it a valuable protein supplement to low quality forage diets (Shelton and Brewbaker 1994; Soedarjo and Borthakur 1996). Leucaena foliage is highly digestible, favored by animals, and a rich source of macro- and micro-elements (Norton 1995). Although highly nutritious, leucaena has an undesirable attribute; it produces a toxic compound, mimosine, which has harmful effects on animals. Because of the toxic effects of mimosine, leucaena can be fed to animals only in limited amounts (<10% of total diet). Among different methods of plant improvement, genetic engineering may be the fastest alternative to resolve the problem of toxicity in leucaena, however an

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efficient transformation protocol is necessary. Another possible benefit of genetic engineering of leucaena is in the field of phytoremediation. Leucaena was found to have the natural ability to uptake and detoxify xenobiotic pollutants from the soil and groundwater (Doty et al. 2003). However, it lacks the catabolic pathways for complete breakdown of these compounds compared to microorganisms. Hence, transfer of genes involved in xenobiotic degradation from microbes to leucaena can further enhance its potential for remediation of harmful compounds.

Tissue culture and plant regeneration of leguminous woody trees have been well established, including in leucaena (Saafi and Borthakur 2002), however, there are only a few reports on the genetic transformation of these plants. Only *Stylosanthes guianensis* (Sarria et al. 1994), *Robinia pseudoacacia* (Igasaki et al. 2000), *Acacia mangium* (Xie and Hong 2002) and *Acacia crassiparpa* (Yang et al. 2008) have been reportedly transformed using *Agrobacterium tumefaciens*. Although Rastogi and Dwivedi (2006) described a transformation protocol for leucaena using mature nodes and cotyledonary nodes as explant material, we could not replicate their results. One of the main obstacles during genetic transformation of trees is the process of regenerating transformed cells into healthy plantlets. In this paper, we report the development of an *Agrobacterium*-mediated transformation protocol to produce transgenic leucaena plants resistant to the herbicide phosphinothricin, using immature zygotic embryo segments of green seeds as the explant material.

Materials and methods

Plant regeneration from immature zygotic embryos

Leucaena cv. K-636 seeds were collected from the University of Hawaii farm in Waimanalo, Honolulu, HI. The immature zygotic embryos were obtained from unripe, 10 weeks post-anthesis seeds that were approximately 9 mm long. The green seeds were thoroughly washed with 1% v/v dishwashing detergent before being surface sterilized with 10% v/v sodium hypochlorite for 10 min with shaking, and washed five times with sterile deionized water. After blot-drying the seeds with sterile filter paper, the testa was removed, exposing the zygotic immature embryo and cotyledons. The cotyledons were removed from the immature embryo and discarded. The embryo was cut in half through its main axis, with each half used as the starting explant material for plant regeneration and transformation.

To reduce oxidative browning of the explant material due to leaching of phenolic compounds into the growth medium, the explants were soaked for 30 min at room

temperature (RT) in antioxidant solution (50 mg l⁻¹ ascorbic acid and 75 mg l⁻¹ citric acid) prior to in vitro culturing. After antioxidant treatment, the explants were kept for 14 days in the dark at 28°C on solid callus induction medium (CIM) containing full-strength MS salts (Murashige and Skoog 1962) and Gamborg vitamins (Gamborg et al. 1968), supplemented with 1 mg l⁻¹ 2,4-dichlorophenoxyacetic acid (2,4-D), 0.5 mg l⁻¹ benzyladenine (BA), 30 g l⁻¹ sucrose, pH adjusted to 5.8 and solidified with 2 g l⁻¹ Phytigel (Sigma-Aldrich, St. Louis, MO). After 14 days, the explants were maintained and cultured in Magenta GA7 boxes (Gibco-BRL, Gaithersburg, MD) with solid shoot induction medium (SIM), containing full-strength MS salts, Gamborg vitamins, supplemented with 3 mg l⁻¹ BA and 0.25 mg l⁻¹ naphthalene acetic acid (NAA), 30 g l⁻¹ sucrose, pH 5.8 under a 16/8-h photoperiod at 60 μmol m⁻² s⁻¹ light intensity, until shoots reached 5 cm in length. Shoots developed from these explants were then transferred to Magenta GA7 boxes with solid root induction medium (RIM) containing half-strength MS salts, Gamborg vitamins, supplemented with 2 mg l⁻¹ indole butyric acid (IBA), 0.1 mg l⁻¹ kinetin, and 30 g l⁻¹ sucrose.

Selective agent

To identify an appropriate selective agent and its concentration to be used in the genetic transformation of leucaena, explants were grown for 30 days in SIM, containing either the antibiotic kanamycin or the herbicide phosphinothricin. Kanamycin was used in a range of concentrations starting at 25 mg l⁻¹ up to 250 mg l⁻¹ in increments of 25 mg l⁻¹; while phosphinothricin was used in concentrations starting at 1 mg l⁻¹ up to 10 mg l⁻¹ in increments of 1 mg l⁻¹.

Binary vector and bacterial strains

We tested the T-DNA transfer ability of three different *Agrobacterium tumefaciens* strains, the agropine strain EHA105 (Hood et al. 1993), the octopine strain LBA4404 (Hoekema et al. 1983), and the nopaline strain C58C1 (Ashby et al. 1988) carrying the plant binary vector pCAMBIA3201 (CAMBIA, Canberra, Australia). This binary vector contains the *bar* selectable marker gene, encoding the herbicide-degrading enzyme phosphinothricin aminotransferase (PAT), and the *uidA* reporter gene, encoding the enzyme β-glucuronidase (GUS), both under the control of the constitutive cauliflower mosaic 35S virus (35S CaMV) promoter and the nopaline synthase (NOS) terminator (Fig. 1). The binary vector was transferred into the *A. tumefaciens* strains using the electroporation method described by Lin (1995).

The *A. tumefaciens* strains were cultured in a shaker (250 rpm) at 28°C for 12 h in 10 ml liquid YEP medium

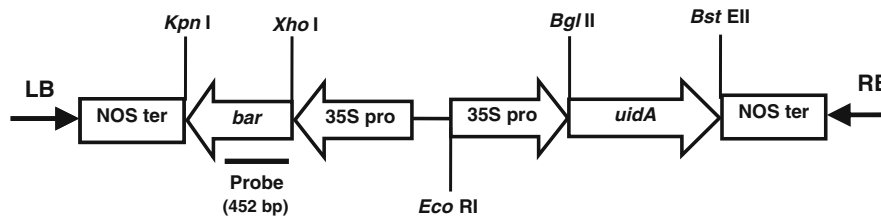


Fig. 1 Diagrammatic representation of the binary plasmid vector pCambia3201 T-DNA region indicating the localization of the *bar* gene, encoding phosphinothricin aminotransferase (PAT) and the

uidA gene, encoding β -Glucuronidase (GUS). LB (left border), RB (right border), NOS ter (nopaline synthase terminator), 35S pro (cauliflower mosaic virus 35S promoter)

(Sambrook et al. 1989), containing 10 mg l^{-1} rifampicin and 25 mg l^{-1} chloramphenicol. After 12 h, an optical density (OD_{600}) of 0.8–1.0 was obtained, measured with a Beckman Coulter DU530 photometer (Fullerton, CA). The *A. tumefaciens* cultures were then centrifuged at 10,000 rpm at 4°C for 10 min and the pellet re-suspended in liquid CIM supplemented with $200 \mu\text{M}$ acetosyringone.

Plant transformation

The immature embryo-derived explants were pre-cultured for 4 days in CIM at 28°C in the dark, then vacuum-infiltrated at 400 mm Hg pressure for 30 min with the *A. tumefaciens* inoculums, washed five times in sterile deionized water, blotted dry on sterile filter-paper and placed on solid CIM supplemented with $200 \mu\text{M}$ acetosyringone for 10 days at 28°C in the dark and 5 days at 28°C with a 16/8-h photoperiod ($60 \mu\text{mol m}^{-2} \text{ s}^{-1}$). This co-cultivation period allowed for the formation of callus tissues and multiplication of the agrobacteria. Following co-cultivation, the explants were cultured for 90 days (transferred to fresh medium every 15 days) in SIM, supplemented with 250 mg l^{-1} cefotaxime, for the elimination of *A. tumefaciens*, and 3 mg l^{-1} phosphinothricin, for the selection of transformed tissues. The surviving explants were then subcultured for another 60 days (transferred to fresh medium every 15 days) on SIM supplemented with 3 mg l^{-1} phosphinothricin for continued selective pressure, without cefotaxime, under a 16/8-h photoperiod at $60 \mu\text{mol m}^{-2} \text{ s}^{-1}$ light radiation from cool-white fluorescent tubes. Regenerating putative transgenic shoots were cut off the callus and rooted on RIM supplemented with 3 mg l^{-1} phosphinothricin until the formation of well developed roots, usually within 30 days. Rooted putative transgenic plants were then transferred to pots and grown in a controlled environmental chamber (R. W. Smith & Co., San Diego, CA).

PCR analysis

Using the method of Lin et al. (2001), 100 mg of leaves from young transformed leucaena plants were used for

isolation of total genomic DNA. PCR reaction mixture was composed of $5 \mu\text{l}$ $10\times$ buffer, $3 \mu\text{l}$ 25 mM MgCl_2 , $1 \mu\text{l}$ 10 mM dNTP mixture, $1 \mu\text{l}$ $20 \mu\text{M}$ each of both forward and reverse primers, 1 unit of Taq-DNA Polymerase (Promega, Madison, WI) and 100 ng of DNA template for a $50 \mu\text{l}$ reaction. PCR was carried out in a GeneAmp PCR system 2700 (Applied Biosystems, Foster City, CA) starting with a denaturing step at 95°C for 10 min, followed by 30 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 1 min 30 s, and finishing with an extension step at 72°C for 5 min. PCR products were separated on a 1% agarose gel using electrophoresis, stained with ethidium bromide and photographed. The primer set used for DNA amplification of a 452 bp segment of the *bar* gene was $5'$ -ATGA GCCCAGAACGACGCC- $3'$ and $5'$ -TCAAATCTCGGTG ACGGGCAG- $3'$.

RT-PCR analysis

A QIAGEN RNeasy plant kit (Valencia, CA) was used for the isolation of total RNA, which was treated with RNase-free DNase (Promega) to remove contaminating DNA before reverse transcriptase PCR (RT-PCR) analysis. The RT-PCR reaction was done according to Promega's Reverse Transcription System protocol. For the synthesis of first-strand cDNA, $1 \mu\text{g}$ of total RNA was incubated at 70°C for 10 min, and used in a reaction containing $4 \mu\text{l}$ 25 mM MgCl_2 , $2 \mu\text{l}$ $10\times$ RT-buffer, $2 \mu\text{l}$ 10 mM dNTP mixture, $0.5 \mu\text{l}$ ribonuclease inhibitor, 15 units of AMV reverse transcriptase, $0.5 \mu\text{g}$ random primers, and nuclease-free water to a final volume of $20 \mu\text{l}$. The reaction was incubated at room temperature for 10 min, 42°C for 15 min, 95°C for 5 min, and kept on ice for 5 min. From the first-strand cDNA reaction, $2 \mu\text{l}$ was used as the template for further PCR amplification of double-stranded DNA. The set of primers used for RT-PCR was the same used for PCR analysis.

Southern blot analysis

Ten μg of plant genomic DNA were digested with *EcoRI* (Promega), separated on an 1% agarose gel, and transferred

overnight with salt transfer buffer ($20\times$ SSC) to a positively charged nylon membrane (GeneScreen *Plus* NEN, Boston, MA) by capillary blot according to manufacturer's instruction. The probe was obtained by labeling a PCR-amplified 452 bp fragment of the *bar* gene from pCAM-BIA3201 with Digoxigenin-11-dUTP using the DIG High Prime DNA Labeling and Detection Starter Kit I from Roche Applied Science (Mannheim, Germany). The probe was denatured by heating in boiling water for 10 min and quickly cooling in ice-water. The denatured probe was hybridized to the membrane overnight at 42°C with gentle agitation in DIG Easy Hyb buffer (Roche Applied Science). The membrane was then washed twice for 5 min at RT in $2\times$ SSC, 0.1% (w/v) SDS, and twice for 15 min at 68°C in $0.5\times$ SSC, 0.1% SDS with constant agitation. Immunological detection was performed using anti-digoxigenin-AP conjugate and visualized with the colorimetric substrates NBT/BCIP according to manufacturer's instruction.

Western analysis

Leaf tissue (100 mg) was frozen in liquid nitrogen and ground with mortar and pestle. The resulting powder was immediately added to 1.0 ml of ice-cold extraction buffer (150 mM NaPO_4 (pH 7.0), 10 mM β -mercaptoethanol, 0.1% Triton X-100, 10 mM Na_2 EDTA (pH 8.0), 0.1% Sarkosyl, 100 mg/ml PVPP). After homogenization of the tissue, extracts were centrifuged at 12,000 rpm for 20 min at 4°C , and the supernatants were placed in Eppendorf tubes and stored at 4°C . Protein concentrations of the crude extracts were estimated by the method of Bradford (1976) with a kit supplied by Bio-Rad laboratories (Richmond, CA). Fifty μg of total proteins isolated from leaves of transformed leucaena were separated in 10% polyacrylamide gels supplemented with sodium dodecyl sulfate (SDS-PAGE), and transferred to a $0.45\ \mu\text{m}$ nitrocellulose membrane (Bio-Rad). Immunodetection was carried out with anti-PAT polyclonal antibody (Sigma) at a 1:3,000 dilution in TBS (100 mM Tris-Cl (pH 7.4) and 0.9% (w/v) NaCl). Western analysis was performed with the 'WesternBreeze' blotting system from Invitrogen (Carlsbad, CA) according to manufacturer's instructions.

Fluorometric GUS assay

The fluorometric assay for specific GUS enzyme activity of transformed leucaena plants was conducted using 4-methyl umbelliferyl glucuronide (MUG) as the substrate, according to protocols established by Jefferson et al. (1987) with some modifications (Cote and Rutledge 2002). The MUG assays were conducted in triplicates for each crude extract

(including an extract from a wild-type leucaena for negative control), using microtiter plates. Fifty microliters of crude extract were added to 450 μl of assay buffer (1 mM MUG), incubated at 37°C in the dark, and 100 μl aliquots were removed at time zero and at 30-min intervals for 120 min and added to 900 μl stop buffer (0.2 M Na_2CO_3) to terminate the reaction. Aliquots (200 μl) from each sample were placed in Eppendorf tubes and visualized under UV light. The same volume was placed into the wells of a microtiter plate and the fluorescence was read by using excitation at 360 nm and emission at 460 nm with a FLX800 Microplate Fluorescence Reader (BioTek Instruments, Winooski, VT). To calibrate the fluorometer, six 4-methyl umbelliferone (MU) standards (0 nM, 100 nM, 250 nM, 500 nM, 750 nM and 1,000 nM) were prepared in stop buffer and measured for the establishment of a standard curve. The GUS activity of each transformant was calculated from the slope of the line showing the increase in fluorescence per minute and was expressed in nanomoles of MU produced per minute per milligram of protein by extrapolating from the MU standard curve.

Herbicide leaf spraying assay

Eight-month-old transgenic leucaena plants expressing the PAT protein and a non-transformed (control) plant were analyzed for their resistance to the herbicide phosphinothricin by the leaf spraying assay (Zaragoza et al. 2004). The leaves were sprayed to run-off (approximately 3 ml) with an aqueous solution of the commercial herbicide formulation (Finale, Bayer) diluted to contain $200\ \text{mg l}^{-1}$ of the active compound phosphinothricin. The tolerance of plants to the herbicide was evaluated visually 5 days after application.

Results

Selective agent

Although the higher concentrations of kanamycin slowed down the development and regeneration of immature embryos, leucaena K-636 was found to be highly tolerant to kanamycin, and the regeneration of shoots from the explants was not completely arrested even at a kanamycin concentration of $250\ \text{mg l}^{-1}$. For this reason, we used the herbicide phosphinothricin as an alternative selective agent. Based on physical observations of the explants, including overall health, formation of tissue necrosis, and arrest of shoot and callus development, it was determined that $3\ \text{mg l}^{-1}$ would be the ideal concentration of herbicide to be used as a selective agent for our protocol.

Plant regeneration and transformation

Our observations showed that the combined effects of the plant growth regulators BA and NAA were essential for shoot regeneration from immature embryo-derived explants, with higher concentrations of BA (3 mg l^{-1}) and lower concentrations of NAA (0.25 mg l^{-1}) giving the highest frequency of shoot formation (Table 1). Root development from the immature embryo-derived shoots was tested under different concentrations of IBA and kinetin. After the regeneration and establishment of primary shoots, they were removed from the explants and transferred to RIM supplemented with 2 mg l^{-1} IBA plus 0.1 mg l^{-1} kinetin. At this hormonal concentrations, 78% of the shoots produced roots within 4 weeks (Table 2). Our experiments also revealed that the pre-culture of the explants on CIM prior to *Agrobacterium* co-cultivation and their maintenance on this medium in the dark for another 10 days allowed for the formation of phosphinothricin-resistant shoots. No phosphinothricin-resistant shoots developed in the absence of the pre-culture and callus induction steps, when the explants were placed directly on SIM after *Agrobacterium* co-cultivation. It seems however that the transgenic shoots originated from the cells of the immature zygotic embryo, and not from the cells of the callus. The callus formed during this stage may have played a role in enhancing *Agrobacterium* transformation by providing a protective layer to the emerging phosphinothricin-resistant shoots.

Three different *A. tumefaciens* strains, EHA105, LBA4404, and C58C1, were used to determine the best-suited

Table 1 The effect of BA and NAA on shoot formation from immature zygotic embryo-derived explants of leucaena

Growth regulator (mg l^{-1})	Shoot frequency (%)
BA	
1	45 ± 3.5
2	41 ± 1.7
3	58 ± 5.3
NAA	
0.05	40 ± 2.0
0.25	43 ± 5.8
0.50	32 ± 3.5
BA + NAA	
3 + 0.05	65 ± 1.6
3 + 0.25	72 ± 3.7
3 + 0.50	61 ± 2.5

Immature embryo-derived explants were cultured on callus induction media (CIM) for 2 weeks then the explants were transferred to shoot induction media (SIM) in the light. Shoot frequency (%) was recorded after 4 weeks on SIM. Fifty immature embryo-derived explants were used in each experiment. Values are the means \pm SD of two experiments

Table 2 The effect of IBA and kinetin on root formation from immature zygotic embryo-derived shoots of leucaena

Growth regulator (mg l^{-1})	Root frequency (%)
IBA	
1.00	35 ± 2.2
2.00	59 ± 5.1
3.00	42 ± 4.7
Kinetin	
0.10	48 ± 7.0
0.25	34 ± 3.6
0.50	39 ± 2.3
IBA + Kinetin	
2.00 + 0.25	55 ± 1.4
2.00 + 0.50	65 ± 3.9
2.00 + 0.10	78 ± 3.3

Immature embryo-derived shoots were cultured on shoot induction media (SIM) for 5 months then the explants were transferred to root induction media (RIM) in the light. Root frequency (%) was recorded after 4 weeks on RIM. Twenty immature embryo-derived shoots were used in each experiment. Values are the means \pm SD of two experiments

strain for generating transgenic leucaena from immature zygotic embryos. A total of 300 explants in one experiment (100 explants per *Agrobacterium* strain) were co-cultivated with the three *A. tumefaciens* strains, and this experiment was repeated twice. After *A. tumefaciens* infection and selection in phosphinothricin-supplemented medium, no shoots were developed from the explants infected with either LBA4404 or EHA105; however, a number of explants infected with the strain C58C1 survived under selective pressure and developed shoots. After multiple transfers, a total of four phosphinothricin-resistant shoots were obtained from immature zygotic embryos infected with strain C58C1. These shoots survived multiple passages in selective medium and were also able to develop roots and be successfully transplanted to pots containing soil (Fig. 2). After 8 months of growth in a controlled environmental chamber, four stable transformants (one from the first experiment and three from the second experiment) were obtained from a total of 200 explants, giving on average, two transgenic lines from 100 immature embryo-derived explants. The overall transformation efficiency of leucaena using the described method was therefore established at 2%. All four transformants showed normal growth and phenotype.

Analysis of transformants

The four putative leucaena transformants were analyzed for the presence and expression of the *bar* gene through PCR, RT-PCR and Western blotting. PCR amplification of DNA extracted from leaves of the transgenic plants using

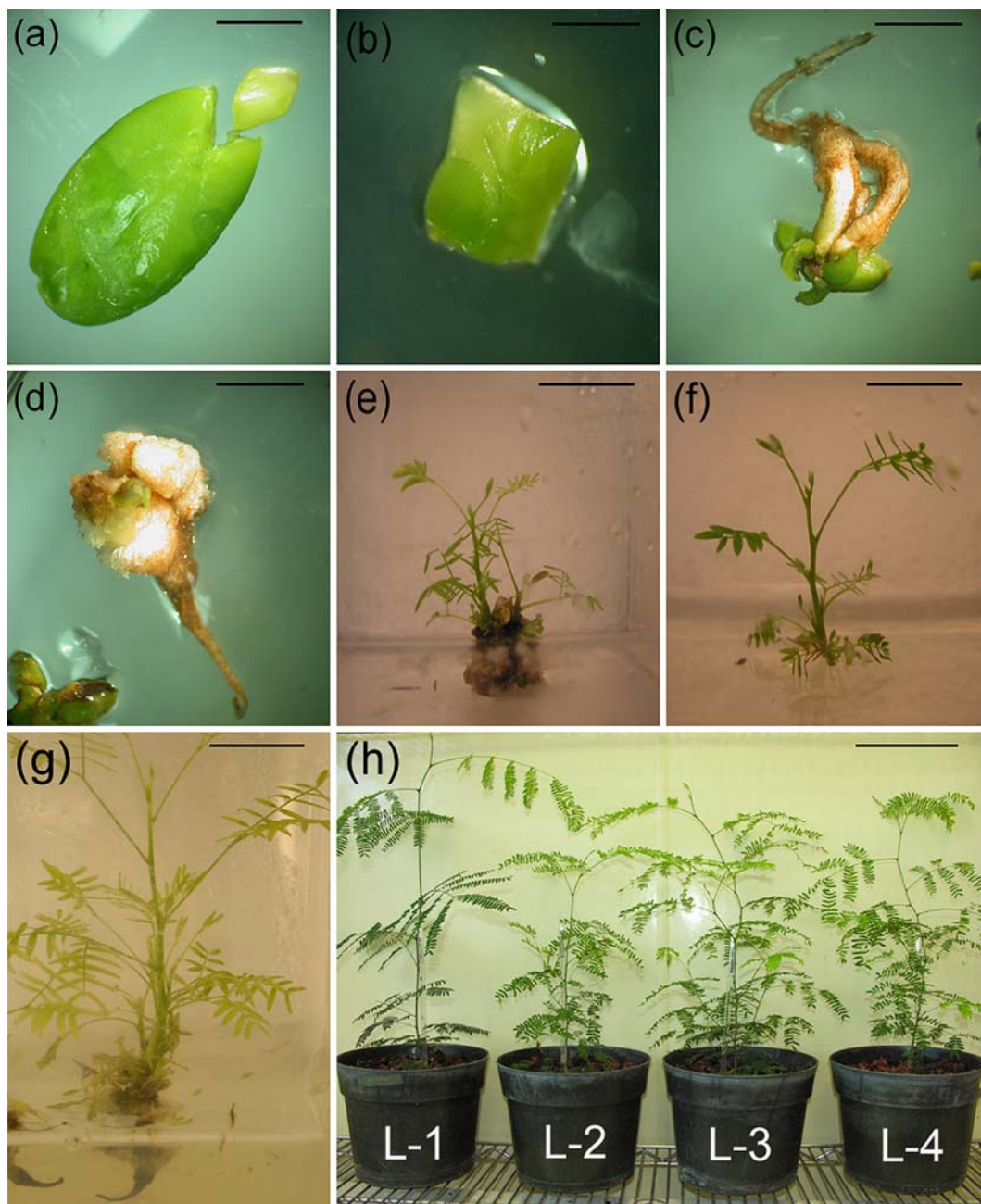


Fig. 2 Transformation and regeneration of leucaena. **a** Excision of zygotic immature embryos from green seeds (bar = 3 mm). **b** Embryos were cut through main axis (bar = 2 mm). **c** Elongation step of split embryos (bar = 2 mm). **d** Callus initiation from dissected embryos in CIM supplemented with 1 mg l^{-1} 2,4-D and 0.5 mg l^{-1} BA (bar = 2 mm). **e** Shoot formation from callus tissue in

SIM containing 3 mg l^{-1} BA, 0.1 mg l^{-1} NAA and 3 mg l^{-1} phosphinothricin (bar = 15 mm). **f** Shoots excised from callus tissue and placed in selective medium (bar = 15 mm). **g** Root development from herbicide-resistant shoots (bar = 15 mm). **h** Eight-month-old transformed plants (bar = 0.2 mm)

bar-specific primers resulted in a 452-bp band that indicates the presence of the transgene (Fig. 3a). Wild-type leucaena plants were used as a control and did not produce bands when analyzed by PCR. RT-PCR analyses using total RNA extracted from leaves of the transformed plants confirmed the presence of the *bar* transcripts (Fig. 3b). The expression of the *bar* genes in the RT-PCR positive

transformed leucaena plants was further confirmed by Western blot analyses using polyclonal antisera raised against the PAT protein. Western analyses revealed the presence of a single band with a molecular mass of 21 kDa in all four transformed plants (Fig. 3c). Southern hybridization confirmed stable integration of the T-DNA in the genome of the four transgenic plants, showing at least one

Fig. 3 Molecular analyses of four transgenic leucaena plants expressing the *bar* gene. **a** PCR analyses show that the four herbicide-resistant transformants contained the *bar* gene (452 bp) **b** RT-PCR analyses show that all four plants produced the *bar* transcript **c** Western analyses show that the transgenic plants produced a 21-kDa PAT protein. The lanes 1, 2, 3 and 4 represent four independently transformed lines. WT—wild-type (non-transformant negative control)

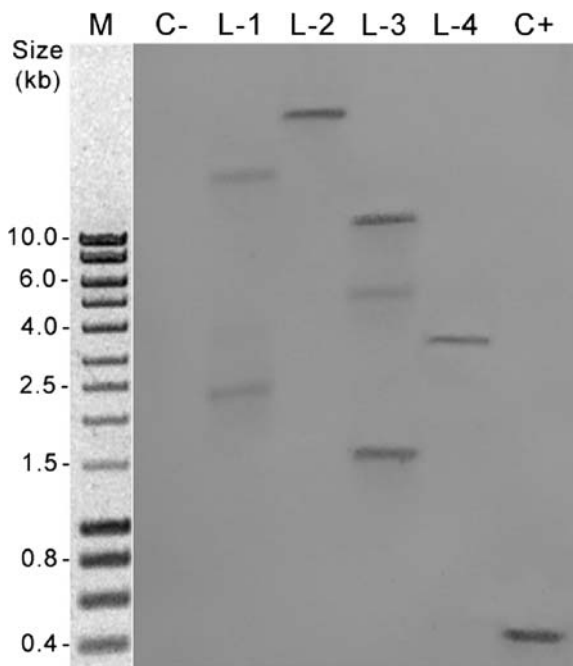
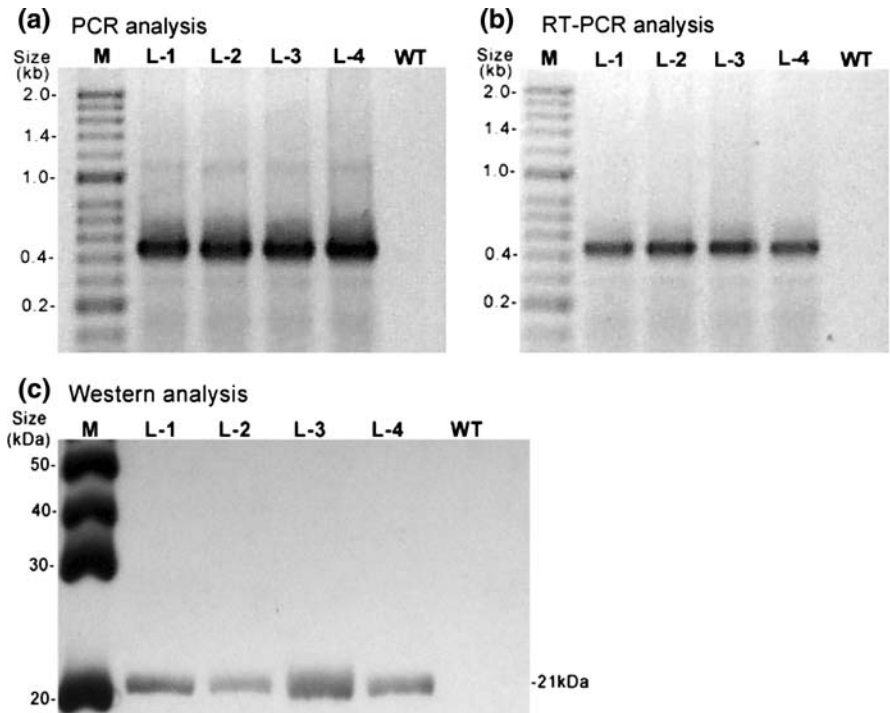


Fig. 4 Southern blot hybridization analysis of genomic DNA extracted from leaves of a non-transformed and four putative leucaena transformants. *EcoRI*-digested DNA was hybridized with a DIG-labeled *bar* probe. M: molecular weight marker; C-: negative control (wild-type); L-1, L-2, L-3 and L-4: four independently transformed lines of leucaena; C+: positive control (PCR-amplified *bar* probe from the pCAMBIA3201 plasmid)

copy of the *bar* gene. The unique banding pattern shown on the Southern blot also suggests that each transgenic plant originated from a separate transformation event (Fig. 4).



Fig. 5 Leaf spraying assay. Leaves from 8-month-old non-transformed (WT) and transformed (L-1, L-2, L-3, L-4) leucaena plants 5 days after herbicide application (200 mg l^{-1} phosphinothricin)

Long-term, stable expression of the *bar* gene was also analyzed by applying the herbicide phosphinothricin to the leaves of the 8-month-old greenhouse-grown transgenic leucaena plants. Within 2 days, necrotic spots appeared on the non-transformed leaves. Five days after application, the transgenic leaflets showed much higher resistance to the herbicide spraying than the leaflets from the non-transformed plant (Fig. 5). In addition to the *bar* gene for resistance to phosphinothricin, the T-DNA of the binary vector pCAMBIA3201 contained the *E. coli uidA* gene, which encodes GUS. Quantitative analyses of the four independently transformed leucaena plants, confirmed the expression of the *uidA* gene in the transgenic plants after 8 months of growth in a greenhouse (Fig. 6).

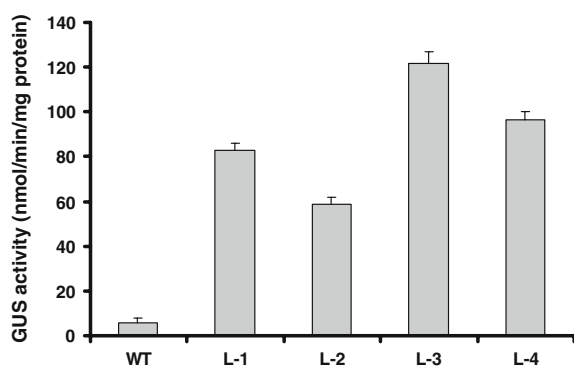


Fig. 6 Fluorometric GUS specific activity in extracts prepared from four 8-month-old leucaena transformants, L-1, L-2, L-3 and L-4. Values represent the mean \pm SD of triplicates. WT—wild-type

Discussion

We report here the development of an *Agrobacterium*-mediated transformation protocol for leucaena, in which immature zygotic embryos were used as the explant material for the generation of transgenic leucaena. During the development of our transformation protocol, we found leucaena to be recalcitrant to genetic transformation by *Agrobacterium*. One of the main obstacles during in vitro culture and plant regeneration of leucaena was the exudation of phenolic compounds by wounded tissues. This caused oxidative browning and subsequent necrosis of the tissues, hindering organogenesis and inhibiting further development of the explants into plantlets.

A number of different explant materials have been previously used in the development of *Agrobacterium*-mediated transformation protocols for woody plants (Archilletti et al. 1995; Le et al. 1996; Franche et al. 1997; Gartland et al. 2000; Bishop-Hurley et al. 2001; Corredoira et al. 2004; Polin et al. 2006; Li et al. 2007). More specifically, for the transformation of leguminous trees, stems from rejuvenated shoots and leaves were used as explants for *A. mangium* and *R. pseudoacacia*, respectively (Xie and Hong 2002; Igasaki et al. 2000). Saafi and Borthakur (2002) developed an in vitro regeneration protocol for leucaena using hypocotyl and cotyledon segments as the start explant material. Although that study was successful in developing a plant regeneration method for leucaena, we were not able to obtain transgenic leucaena plants using hypocotyl- or cotyledon-derived explants. We used different plant regeneration methods and different explant materials to transform leucaena with *A. tumefaciens*. Successful production of transgenic leucaena plants was achieved only with the use of explants derived from immature zygotic embryos. Our success in obtaining transgenic leucaena using immature embryo-derived explants is probably due to the fact that by removing most excess plant material from the embryos and splitting them in halves, we increased the exposure of

actively dividing plant cells to *A. tumefaciens*, facilitating infection and subsequent transfer of the T-DNA into the plant cell. It has been previously shown that host-cell division is required for successful *Agrobacterium* transformation (Binns and Thomashow 1988).

The use of vacuum infiltration during co-cultivation of explants with *A. tumefaciens* also proved to be essential for the successful development of transformed leucaena. In previous trials in our laboratory, no transformants were obtained without the use of vacuum (data not shown). This observation is consistent with other studies that found the use of vacuum infiltration to be important in increasing transformation efficiency in woody plants (Charity et al. 2002). Transformation efficiency often depends on the strain of *A. tumefaciens* used (Hood et al. 1993; Wenck et al. 1999). We tested three different strains (LBA4404, EHA105 and C58C1) for transformation of leucaena. Only co-cultivation with the nopaline strain C58C1 resulted in successful generation of transformed leucaena plants. Igasaki et al. (2000) obtained similar results during *Agrobacterium*-mediated transformation of *R. pseudoacacia*, showing that the nopaline strain GV3101 gave much higher frequency of transformation compared with the octopine strain LBA4404 and the agropine strain EHA101.

Previously, Rastogi and Dwivedi (2006) reported the development of a transformation protocol for leucaena. In their report, they obtained 13–20% transformation efficiency using mature nodes and cotyledonary nodes as the explant material, and kanamycin as the selective agent. After repeated attempts to reproduce their results, we were not successful in generating transgenic leucaena plants. The main obstacle that we encountered when trying to replicate their protocol was that all the leucaena cultivars tested, including the ones used by them (K-8, K-29, and K-850), were highly resistant to kanamycin under our conditions, which made it impossible to detect transformed cells using this selective agent. High resistance to kanamycin was also observed in other leguminous trees such as *A. mangium*, a member of the *Fabaceae* family, which was reportedly resistant to kanamycin up to a concentration of 300 mg l⁻¹ (Xie and Hong 2002) and *R. pseudoacacia*, which was shown to be resistant to the use of kanamycin as a selective agent during the development of an *Agrobacterium*-mediated transformation protocol (Igasaki et al. 2000). We also tested the protocol using the binary plasmid pCAMBIA-3201, which carries the *bar* gene as the selectable marker, and again we could not obtain transformed leucaena plants using explants derived from mature nodes and cotyledonary nodes. Using immature zygotic embryos as explants, co-cultivated with the *Agrobacterium* strain C58C1 and under the selective pressure of the herbicide phosphinothricin, we were able to generate four transgenic leucaena plants, obtaining a transformation efficiency of 2%.

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