academicJournals

Vol. 15(37), pp. 2006-2016, 14 September, 2016
DOI: 10.5897/AJB2016.15458
Article Number: B32654E60520
ISSN 1684-5315

http://www.academicjournals.org/AJB

African Journal of Biotechnology

Full Length Research Paper

Agrobacterium-mediated transformation of *Jatropha curcas* leaf explants with a fungal *chitinase* gene

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Received 9 May 2016; Accepted 7 September, 2016

***Jatropha curcas* L. oil has been shown to be suitable for the production of biodiesel. However, this species has not been domesticated yet. Genetic breeding through conventional methods is time consuming and costly, hence, genetic transformation could contribute positively to the improvement of interesting traits. Although *in vitro* regeneration and stable genetic transformation has been pursued for several years, variation in transformation efficiency remains strongly genotype-dependent and indicates that protocols optimization is still needed. Thus, this study was carried out to introduce a *chitinase* gene from the *Trichoderma viride* fungus into the genome of a *J. curcas* superior genotype by inoculating leaf explants with *Agrobacterium tumefaciens* EHA 105 strain in axenic conditions. Some key parameters such as pre-culture period and antibiotic doses were optimized with 500 mg.L^{-1} cefotaxime and 100 mg.L^{-1} kanamycin concentrations being suitable for *A. tumefaciens* inhibition and explant selection, respectively. The best transformation efficiency (50%) was obtained when leaf explants were incubated on a culture medium promoting shoot regeneration at 15 days before the induction of the transformation process. Plants where *chitinase* gene amplicons could be detected were assessed for transgene copy number and expression levels by quantitative real-time polymerase chain reaction (PCR). One, two and three copies of the introduced gene were confirmed in nine transgenic lines with two of them that were assessed for gene expression and showed quantitative variation for this variable. These results bring valuable information for further gene insertions in breeding programs of *J. curcas* for fungal disease resistance.**

Key words: *Agrobacterium tumefaciens*, pre-culture time, kanamycin, quantitative real-time polymerase chain reaction (PCR), transgene copy number.

INTRODUCTION

Considering the importance of renewable energy sources to sustain the global energy matrix, biofuels are among the available alternative to mitigate the current energy crises and climate changes (Divakara et al., 2010; Varshney and Johnson, 2010). In Brazil, nowadays, the blend of 7% biodiesel into fossil oil (B7) is already mandatory; it is scheduled to reach 10%, until 2019 and even 20%, until 2030 (Giersdorf, 2013). The progressive increase in commercial diesel blend on the market has resulted in positive environmental, economic and social impact. However, it will also require urgent oil sources diversification to sustain the growing demand for biodiesel.

Due to its high oil content and quality, *Jatropha curcas* L., has been proposed as a promising plant source to diversify biodiesel and biokerosene production in tropical climates (Carels, 2013; Sharma, 2011; Li et al., 2007). Despite its potential, *J. curcas,* also known as physic nut, is a perennial and semi-wild species, which still needs years of selective breeding until the release of a stable cultivar to the world. Plant domestication and genetic improvement process could be sped up by the integration of biotechnology approaches to the conventional breeding process for the introduction of agronomical desirable traits such as oil content and quality, synchronous fruit maturity, early flowering and disease or pest resistance. Although considered as a stress resistant species (Openshaw, 2000; Debnath and Bisen, 2008; Sabandar et al., 2013), physic nut has proven to be highly susceptible to a wide array of pest and disease when it is raised as a monoculture, which may limit its seed and oil yields (Kumar et al., 2013; Sujatha, 2013; Argollo Marques et al., 2013).

The domestication process through selective breeding is a purifying process that leads to the reduction of genetic variability by capturing both additive and non-additive effects in elite clones via selection and clonal propagation (Carels, 2013). Despite some variations in agro-morphological and chemical properties reported in different studies (Wani et al., 2006; Kaushik et al., 2007; Rao et al., 2008; Argollo Marques et al., 2013), only a limited number of traits has been observed in the *J. curcas* genotypes evaluated so far. The major part of these traits involves genes engaged in mechanisms of resistance to biotic and abiotic stresses (Argollo Marques et al., 2013).

The genetic base broadening can be obtained by interspecific hybridization and/or biotechnological approaches. The introgression of stress resistance genes as well as other favorable genes found in wild congeneric species into *J. curcas* may be a promising breeding strategy (Sujatha et al., 2013; Argollo Marques et al., 2013), however, it is time consuming, expensive and limited to the closest wild species (Guidolin, 2003). Furthermore, undesirable genes are inevitably co-inherited and need cycles of backcrossing to be eliminated. In turn, the genetic engineering strategy has many advantages such as the shortening of the number of selective breeding steps and the ability to introduce genes for traits that may not be even available within congeneric species (Visarada et al., 2009; Herr and Carlson, 2013; Fu et al., 2015). However, to be successful, the genetic transformation process depends from the availability of an efficient and reproducible *in vitro* regeneration system. To date, plant regeneration protocols have been developed using various types of explants (Wei et al., 2004; Jha et al., 2007; Deore and Johnson, 2008; Kumar and Reddy, 2010; Kumar et al., 2010; Sharma et al., 2011; Toppo et al., 2012; Franco et al., 2014). Based on these regeneration systems, stable transformation procedures have been developed employing both *Agrobacterium tumefaciens* (Li et al., 2008; Kumar et al., 2011; Pan et al., 2010; Misra et al., 2012; Fu et al., 2015; Nanasato et al., 2015) or particle bombardment (Joshi et al., 2011; Purkayastha et al., 2010).

Due to little transgene re-arrangement, low copy number and preferential integration into transcriptionally active regions of chromosomes, *Agrobacterium*-mediated transformation is the most widely used method to generate transgenic *J. curcas* (Kumar et al., 2013; Fu et al., 2015). Li et al. (2008) first reported *J. curcas* genetic transformation via *A. tumefaciens* infection of cotyledon disc using the phosphinothricin herbicide as selective agent, however, the transformation efficiency was low (13%). Recently, Nanasato et al. (2015) improved the rate of cotyledon transformation efficiency to 23.3% using vacuum infiltration for the step of bacterial inoculation and co-culture on filter-paper wicks instead of agar for the step of latency before shoot regeneration. Fu et al. (2015) further increased the rate of transformation efficiency to 56% by optimizing kanamycin concentration in the selective medium and the duration of the selection process. Despite their higher potential of *in vitro* shoot regeneration, juvenile explants such as cotyledons and hypocotyls may results in a higher rate of unexpected somatic variation (Bhatia et al., 2005). Due to the smaller rate of somatic variation in regenerated shoots obtained

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from sporofitic explants such as stem and leaves, these tissues should be preferred for transformation (Kumar et al., 2010; Misra et al., 2012). Zong et al. (2010) using leaf explants, kanamycin for selection medium and a 2 days pre-culture period, increased the rate of transformation efficiency to 23.91%. Kumar et al. (2010) using hygromycin as another selective agent with leaf explant and a pre-culture period of 4 days achieved an even greater efficiency of 29%. Despite these efforts, large variations among different *Jatropha* genotypes in terms of their morphological responses to phytohormones is still observed *in vitro* and the rate of transformation efficiency of differentiated tissues still varies between 4 and 56% (Basa and Sujatha, 2009; Fu et al., 2015). Thus, the optimization of the regeneration and transformation procedures need to be conducted according to their genetic profile, which makes sense at least for a small number of high-yielding elite genotypes that have been recently selected by conventional breeding programs (Basa and Sujatha, 2009).

Currently, efficient *in vitro* regeneration protocols were developed using foliar explants for cloning elite genotypes selected under the breeding programs of Instituto Agronômico of Campinas (IAC) in Brazil (Franco et al., 2014). High regeneration rates (~40 shoots per explant) and efficient rooting via micrografting (85%) were obtained for the L4P49 genotype. The micro-propagated shoots were considered genetically stable after assessing their ploidy by flow cytometry and their DNA polymorphism by marker-tartrate-resistant acid phosphatase (TRAP), indicating preservation of clonal stability and phenotype uniformity. The subsequent adaptation of this regeneration protocol to an effective and reproducible *Agrobacterium*-mediated transformation protocol with optimization of some key transformation parameters such as pre-culture period and antibiotics concentration was the purpose of the present report. Then, the combined transformation and regeneration protocol was applied to transfer a fungal *chitinase* gene into the genome of a *J. curcas* elite plant to confer fungal disease resistance.

MATERIALS AND METHODS

Plant

Young apical and fully developed leaves were collected from plants of L4P49, a high fruit and oil yielding accession of *J. curcas* selected as an elite genotype by IAC. The leaves were surface sterilized for 15 min with 2.5% sodium hypochlorite (NaClO) solution and rinsed three times with sterile distilled water. Foliar segments (5 × 5 mm) were excised and cultured on a shoot regeneration medium (RM), composed by the salts and vitamins of Murashige and Skoog (1962) and denoted here as MS, which were supplemented with myo-inositol (100 $mg.L^{-1}$), cysteine (10 $mg.L^{-1}$), glutathione (25 $mg.L^{-1}$), sucrose (30 $g.L^{-1}$), hydrolyzed casein (0.5 $g.L^{-1}$), copper sulfate (6 $mg.L^{-1}$), indole-3-butyric acid (IBA, 0.2 $mg.L^{-1}$), thidiazuron (TDZ, 1 $mg.L^{-1}$), and phytagel (2.4 $g.L^{-1}$).

Regenerated shoots were elongated on MS medium supplemented with 6-benzylaminopurine (BAP, 0.3 $mg.L^{-1}$) and IBA (0.1 $mg.L^{-1}$). The pH of all medium was adjusted to 5.8, prior to sterilization (121°C for 20 min). The elongated shoots of 1.5 cm length after 30 days old cultures were used as a source of foliar explants for testing kanamycin resistance for further.

All cultures were maintained at 25 ± 2°C under a 16/8 h (day/night) photoperiod with light provided by cool white fluorescent lamps at an irradiance of 35 to 40 µmol m^{-2} s^{-1}.

Agrobacterium strain and binary vector

A cDNA of the *ech42 chitinase* gene was amplified by quantitative amplification of reversed transcripts by polymerase chain reaction (qRT-PCR) from *Trichoderma viride* grown on media supplemented with colloidal chitin. The *ech42 chitinase* gene was initially cloned in pGEM-T Easy (Promega) and completely sequenced. Subsequently, it was sub-cloned in pCAMBIA 1201 using the NcoI and BstEII restriction sites, substituting the *uidA* (GUS) gene, and transferred to pCAMBIA 2201 using the EcoRI and BstEII sites.

The *ech42* gene is under the control of a CaMV 35S promoter in Pcambia 2201 vector and the resistance to kanamycin for transgenic plant selection is conferred by a *neomycin phosphotransferase II* gene (*nptII*). The pCAMBIA 2201 vector (Figure 1) was introduced by electroporation into the disarmed *A. tumefaciens* EHA105 strain.

A. tumefaciens strain containing a binary vector pCAMBIA 2201 were inoculated in solid LB (Miller, 1972) medium supplemented with kanamycin (100 $mg.L^{-1}$) and rifampicin (100 $mg.L^{-1}$) under 28°C for 72 h. A single colony of the transformed bacteria was isolated and inoculated in liquid LB medium supplemented with kanamycin (100 $mg.L^{-1}$). Bacterial cultures were grown at 200 rpm and 28°C for 16 h. The bacterial suspension was centrifuged at 3,000 rpm for 15 min and the cell pellets were re-suspended in sterile distilled water before further use.

Determination of phytotoxic level of antibiotics

The *A. tumefaciens* EHA105 strain was inoculated in LB medium containing cefotaxime at 0, 300, 400 and 500 $mg.L^{-1}$ and maintained in biochemical oxygen demand (BOD) at 28°C. After five days, the bacterial growth was evaluated in order to determine the minimum dose necessary for total growth inhibition.

The phytotoxic level of kanamycin was determined in order to find the optimal dose that should be used in selective medium. Two types of untransformed foliar explants were cultured separately on RM including an inhibitory dose of cefotaxime previously identified as 500 $mg.L^{-1}$ and kanamycin at 0, 25, 50, 75 and 100 $mg.L^{-1}$. The first type of foliar explants consisted of leaf sections from shoots grown *in vitro* (called *in vitro* leaf explants) and the second was constituted by leaf segment taken from *in vivo* cuttings, grown in a greenhouse (called *in vivo* leaf explants). All cultures were maintained at 25±2°C and 16/8 h (day/night) photoperiod. Both antibiotics were filter-sterilized (Millipore ® 0.22 µm pore size) and added to media after autoclaving. The control medium was RM without antibiotic (cefotaxime and kanamycin). To assess the threshold of kanamycin toxicity, an experimental scheme of five plates per treatment and six explants per plate were used. Three repetitions of the test of kanamycin toxicity assessment were performed independently. The assessment of kanamycin toxicity was obtained through the relative number (%) of bleached explants after six weeks of subculture. The rate of shoot regeneration was calculated as the average number of regenerated shoots per explant.

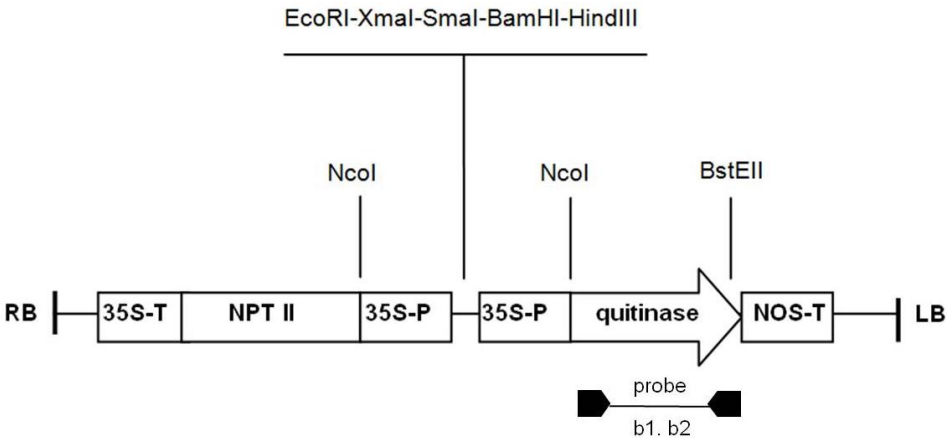

Figure 1. Expression cassette of the vector (pCAMBIA 2201 – *ech 42*). RB: right border of T-DNA; LB: left border of T-DNA; 35S-P: CaMV 35S promoter; 35S-T: CaMV 35S terminator; NOS T: nopaline synthase terminator.

Genetic transformation and effect of pre-culture time

Leaf explants were pre-cultures on RM during 0, 2, 4, 6, 8, 12 and 15 days prior to *Agrobacterium* inoculation in order to find the optimal conditions for the infection process. Explant inoculations were carried out by immersion in a bacterial suspension (OD_{600}=0.8) for 20 min. Then, explants were blotted dry on sterile paper and cultured on solid MS medium (7 $g.L^{-1}$ agar) without antibiotic during three days under total darkness at 24 ± 1°C. Then, explants were transferred to a selective medium, which was RM supplemented with cefotaxime (500 $mg.L^{-1}$) and kanamycin (100 $mg.L^{-1}$), to induce callus and shoot regeneration. Explants were kept for 4 weeks under 16 h photoperiod (35 to 40 µmol m^{-2} s^{-1} irradiance) at 25 ± 1°C and transferred to fresh media every 15 days until shoot regeneration. Shoots that survive on selective medium (with kanamycin) were transferred to solid MS (7 $g.L^{-1}$ agar) with cysteine (10 $mg.L^{-1}$), reduced glutathione (25 $mg.L^{-1}$), hydrolyzed casein (500 $mg.L^{-1}$), copper sulfate (6 $mg.L^{-1}$), adenine sulfate (50 $mg.L^{-1}$), sucrose (30 $g.L^{-1}$), agar (7 $g.L^{-1}$), BAP (0.3 $mg.L^{-1}$), IBA (0.1 $mg.L^{-1}$), and cefotaxime (500 $mg.L^{-1}$). Each treatment included 36 leaf explants.

After six weeks, the rate (%) of kanamycin-resistant shoots that overcame 1.5 cm was calculated relative to the total number of inoculated leaf explants. The transformation efficiency was estimated on the basis of one kanamycin-resistant shoot per explant as the ratio between the number of shoots with *ech42* amplicons (PCR^+) relative to the total number of inoculated explants.

Confirmation of transgenic shoots by PCR

Transformation was confirmed by PCR amplification using total genomic DNA extracted according to Doyle and Doyle (1990). DNA concentration was measured using a Nano Drop spectrophotometer (NanoVue plus-GE). Specific primers for the *ech42* gene (F: 5' CACTTCACCATGTTGGGCTTCCTC 3' and R: 5' GATCTCTAGTTGAGACCGCTTCGG 3') were obtained using Primer3 (Rozen and Skaletsky, 2000) and expected to produce an amplicon of 1.3 Kb. The amplification reaction was conducted on a Bio Rad T100™ thermocycler in a final volume of 25 µl with 50 ng of each DNA sample in 4.0 µl buffer reaction, 1.2 µl $MgCl_2$ (25 mM), 0.4 µl dNTP mix (2.5 mM of each), 0.5 µl of each primer F and R (10 µM), 0.25 µl of Taq DNA polymerase (Thermo Fisher Scientific). The amplification started at 95°C for 4 min, followed by 30 cycles of 30 s at 95°C, 30 s at 62°C and 2 min at 72°C, followed by a final extension of 7 min at 72°C. PCR products were separated on 1% agarose gel electrophoresis (0.5X TBE, 110 V) and visualized on Alpha Imager HP (Cell Biosciences) using GelRed staining.

Estimation of transgene copy number by qRT-PCR

Nine putative transgenic primary shoots were analyzed by qRT-PCR using SYBR green to estimate the number of *ech42* copies inserted in the *J. curcas* genome. Standard curves were prepared for the *ech42* transgene and endogenous *JcKASIII* gene using different dilution of genomic DNA: 75.0, 37.5, 18.75, 9.37, and 4.68 ng. Triplicates of PCR amplifications were performed in a final volume of 20 µl containing: 1 µl DNA, 4 µl primer mix (forward and reverse - 200 nM each), 5 µl H_2O milli Q (Ambion) and 10 µl SYBR® Green master mix (Life Technologies, USA). qRT-PCR experiments were performed using *ech42* primers (F: 5'-AGAACGGTATCTGGGACTACAAGGT-3' and R: 5'-GTAGTACGCCTGTGC GACAGAGT-3'; amplicon size: 80 bp) as well as primers (F: 5' GCACTTGGCTGCAAAACAAAT 3' and *JcKASIII* R: 5' CGTCCAGTCAACATATCGAG 3'; amplicon size: 174 bp) for *JcKASIII*, which is present in a single copy in the *J. curcas* genome according to Jha et al. (2013).

Amplifications were performed using the following program: 55°C for 5 min, 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min, followed by a dissociation step (95°C - 15 s, 60°C - 30 s, and 95°C - 15 s) and repeated twice independently with three replicates each. The estimation of *ech42* copy number was made according to Mason et al. (2002) by comparing its standard curves plotted using threshold cycle (Ct) values relative to those of *JcKASIII* in control and transgenic plants. The r1 coefficient (virtual calibrator) was calculated for *ech42* using data from all transgenic and control plants, as discussed by Mason et al. (2002).

Analysis of gene expression using qRT-PCR

For gene expression analyses, total RNA was extracted from two

Table 1. Effect of antibiotics (cefotaxime and kanamycin) on explants of *J. curcas* after six weeks of subculture.

Explant type/treatment	Bleaching (%)	Callus (%)	Shoots (%)	Number of shoots per explants[1]
In vitro				
Control	15.6	100.0	41.6	1.8^{cd}
T1 (C^2 +$K^3$0)	17.8	100.0	63.0	3.9^{bc}
T2 (C + K25)	81.5	94.9	89.5	9.1^{a}
T3 (C+K50)	96.1	77.0	72.6	7.1^{a}
T4 (C+K75)	98.9	51.3	48.5	4.6^{b}
T5 (C+K100)	94.6	16.3	13.1	0.6^{d}
In vivo				
Control	58.8	100.0	82.5	4.4^{AB}
T1 (C^2 +$K^3$0)	32.0	100.0	76.0	13.0^{A}
T2 (C + K25)	53.3	100.0	100.0	3.2^{B}
T3 (C+K50)	43.0	31.5	23.0	1.3^{B}
T4 (C+K75)	64.0	60.0	34.0	2.0^{B}
T5 (C+K100)	84.0	14.0	10.0	0.2^{B}

[1]Values with different letters for the same explant are significantly different ($P < 0.05$, Tukey's test), CV=52.47%. [2]C: Cefotaxime (500 mg.L^{-1}). [3]K: Kanamycin doses with 0 (K0); 25 (K25); 50 (K50); 75 (K75) and 100 mg.L^{-1} (K100).

independent PCR^+ shoots randomly selected and amplified in quadruplicates and one non-transgenic plant (negative control) using PureLink™ RNA mini kit (Ambion). The RNA was quantified with a NanoDrop spectrophotometer (NanoVue plus-GE). The first-strand cDNA was synthesized according to Super Script III First Strand synthesis (Invitrogen). qRT-PCR reactions were performed using: 4 µl cDNA (10 ng/µl), 10 µl SYBR Green, 4 µl forward and reverse primers mix (final concentration 200 nM each) and 2 µl Milli-Q water. A total of four primers pairs were tested: three for *ech42* (pr1, pr2 and pr3) and one for the internal control (*actin* gene) as described subsequently: pr1 – F: 5' TGCCTACGCCGATTATCAGAA 3' and R: 5' TGCTTCACACAGCCGTATGC 3' (amplicon size: 81 bp), pr2 – F: 5' AGAACGGTATCTGGGACTACAAGGT 3' and R: 5' GTAGTACGCCTGTGCGACAGAGT 3' (amplicon size: 80 bp), pr3 – F: 5' AACGCATACGGCTGTGTGAAG 3' and R: 5' GCCACCGATAGAGAGCATAACCT 3' (amplicon size: 81 bp), *actin* gene – F: 5' GAACTGGAATGGTGAAGGCT 3' and R: 5' ACATAGGCATCCTTCTGACC 3' (amplicon size: 124 bp).

qRT-PCR reactions were carried out in the same conditions as described earlier. The expression levels were determined using the ΔCt and $2^{\Delta\Delta Ct}$ methodology (Bookout, 2003).

Statistical analysis

Experimental data were subjected to Tukey's test ($p < 0.05$) todetermine the statistical significance of the differences among the means using the Sanest Software (Machado and Zonta, 1995).

RESULTS

Antibiotic effects on explants and shoots in *in vitro* culture

Sensitivity tests revealed that the *Agrobacterium* growth was completely inhibited by 500 mg.L^{-1} cefotaxime but lower concentrations did not prevent its overgrowth; consequently, this dose was used in posterior transformation experiments.

The effect of cefotaxime (500 mg.L^{-1}) and increasing kanamycin doses was assessed separately on leaf explants from *in vitro* and *in vivo* sources. Cefotaxime seems to stimulate shoots regeneration in both explant kinds. *In vitro* leaf explant showed higher percentage of shoots production (63%) and shoots per explant (3.9) compared to control (without cefotaxime) in which shoots production was 41.6% and shoots per explant was 1.8. Similarly, the number of shoots per *in vivo* explants increased from 4.4, in the control, to 13.0 upon cefotaxime addition (Table 1).

The phytotoxic level of kanamycin was 100 mg.L^{-1} in both type of explants with a rate of shoots production almost nil after 30 days of culture: 0.6 shoots per *in vitro* leaf explant and only 0.2 shoots per *in vivo* leaf explant. Explants started to bleach after 15 days subculture, resulting in the total loss of chlorophyll (bleaching) in approximately 95 and 84% of *in vitro* and *in vivo* leaf explants, respectively (Table 1). After 30 days on kanamycin medium, all shoots became necrotic and started to die. Based on these results, RM was supplemented with 100 mg.L^{-1} of kanamycin for effective selection of transformed shoots.

Genetic transformation and effect of pre-culture time

EHA strain of *A. tumefaciens* was efficient for infecting foliar explants of *J. curcas* superior genotype L4P49

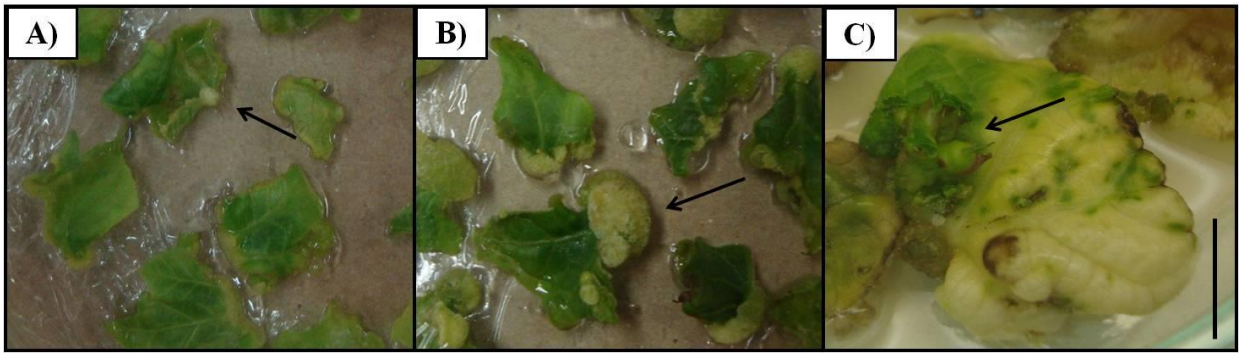

Figure 2. Aspect of explants precultured in regeneration medium (RM) during 6 (a) and 15 days (b). Arrows indicate the onset of callus formation. (c) Shoot regeneration observed after explant co-cultivation with *A. tumefaciens*. Putative transformed explants were kept on RM supplemented with 100 mg.L^{-1} of kanamycin. Bar=1 cm.

Table 2. Effect of pre-culture period on shoot regeneration and transformation efficiency.

Pre-culture period (days)	Inoculated explants	Kan^R shoots[(a)]	Regeneration rate[(b)]	PCR$^{(+)}$ shoots[(c)]	Transformation Efficiency (%)[(d)]
6	36	280	7.8	12	33.3
8	36	122	3.4	10	27.8
12	36	201	5.6	16	44.4
15	36	254	7.0	18	50.0

[(a)]Kan^R shoots (kanamycin-resistant shoots): Number of shoots that regenerated on selective medium containing kanamycin (100 mg.L^{-1}). [(b)]Regeneration rate: Kan^R shoots/total number of inoculated leaf explants. [(c)]PCR$^{(+)}$ shoots: Number of independent Kan^R shoots (one Kan^R shoot from each different explant) with positive result after PCR amplification. [(d)]Transformation efficiency (%): (number of PCR$^{(+)}$ shoots/total number of inoculated explants) × 100.

using bacterial suspension of 0.8 OD_{600}, three days co-culture period and suitable selective medium (RM medium supplemented with 500 mg.L^{-1} cefotaxime and 100 mg.L^{-1} kanamycin). Leaf explants directly infected with *A. tumefaciens* (without pre-culture) did not show any transformation and those pre-cultured on RM for two to four days before bacterial infection showed only low regeneration rate with few incompletely developed shoots unsuitable to the transformation efficiency assessment. Pre-culture time larger than six days allowed the onset of callus on the foliar explant edges (Figure 2). The explants pre-cultured on RM for 12 to 15 days prior to co-cultivation with *A. tumefaciens* gave the highest rate of callus formation resulting in larger rate of kanamycin-resistant shoot production on selective medium (Table 2). The largest transformation efficiency (50% of explants responded positively to transformation procedure) was observed when explants were pre-cultured on RM for 15 days with an average of seven kanamycin-resistant shoots per explant (Table 2).

Confirmation of transgenic shoots and estimation of transgene copy number

PCR analyses confirmed the presence of a 1.3 kb fragment equivalent to the expected amplicon size of the *ech42* gene into independent primary shoots grown on selective medium (Figure 3).

Standard curves of Figure 4 show the reaction efficiency and Ct values of each primer pairs for qRT-PCR assays as well as for calculation of copy number ratios. The evaluated amplification efficiency for the target gene (*ech42)* and for the endogenous reference gene (*JcKASIII*) was over 100% (Figure 4). The virtual calibrator (r_1 coefficient) calculated for *ech42* as described by Mason et al. (2002) was 0.9. Thus, the estimated *ech42* to *JcKASIII* ratios gave measures of the number of *ech42* copies effectively inserted in the *J. curcas* genome of each transformed plant (Table 3). Single, double and triple copies of the *ech42* gene were found in the transgenic lines evaluated.

Measures of gene expression by qRT-PCR

The experimental values of dissociation curves for *chitinase* and *JcKASIII* genes overlapped the adjusted regression lines, which demonstrate the reliability of our results. A single peak was observed in the dissociation curves, confirming the amplification of only one fragment

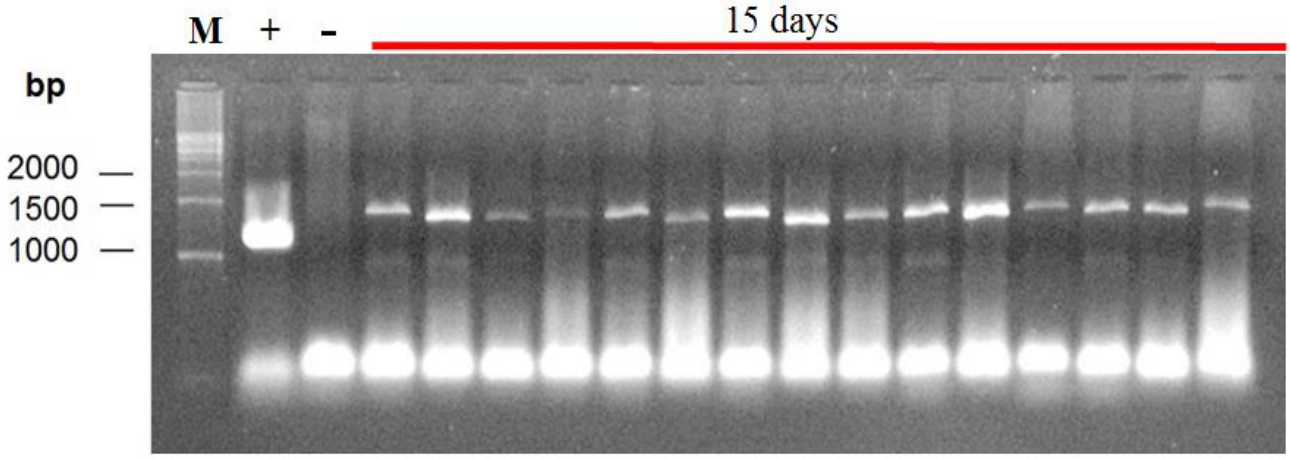

Figure 3. PCR analysis of 1000 and 1500 bp amplicons of the *ech 42* gene in transgenic plants. Lanes: *M* molecular size marker (1 Kb ladder), lane "+": positive control (plasmid DNA), lane "-": negative control (non-transgenic plant), red bar: Amplicons in putative transgenic plants from 6, 12 and 15 days pre-culture on RM.

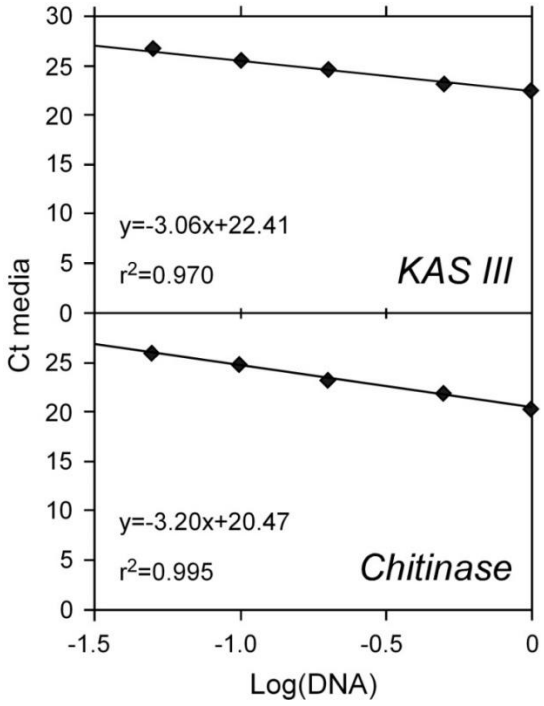

Figure 4. Plot of standard regression lines (Ct media) of *JcKASIII* (internal control) and *chitinase* genes (target) for quantities of genomic DNA corresponding to 75, 37.5, 18.75, 9.37, and 4.68 ng (Log(DNA)). Reaction efficiency percentages for *JcKASIII* and *chitinase* were 112.74% and 105.35%, respectively.

with all primers combinations analyzed for both primary shoots #12 and #23. In these two particular cases, it was found out that qRT-PCR allowed the differentiation of transgene expression levels by reference to the *actin* gene used as a control. Actually, the level of *ech42* expression observed in the #12 plant was 2 times larger than that of *actin* gene whereas it was 168 time larger than this internal control in the # 23 plant (Figure 5).

Table 3. Estimated and assumed copy number for *chitinase* gene in nine PCR$^+$ shoots.

Plant	Estimated copy number	Assumed copy number
1	0.86	1
2	1.04	1
3	1.64	2
4	0.83	1
5	0.90	1
6	0.28	0
7	0.24	0
8	0.76	1
9	2.76	3

DISCUSSION

Agrobacterium-mediated transformation requires previous determination of specific antibiotics at suitable concentration to completely inhibit bacterial growth without affecting shoot regeneration. Beta-lactam antibiotics, such as cefotaxime, are the most commonly used antibiotics in plant transformation protocols, since

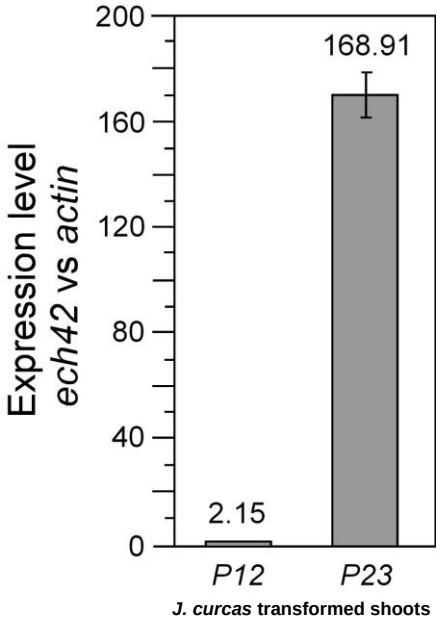

Figure 5. Expression levels of *ech42* in two *J. curcas* transformed shoots: Plant #12 and Plant #23 in relation to the *actin* gene of *J. curcas.*

they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes (Yu et al., 2001). Although inhibitory effect on shoot regeneration have already been previously reported (Tang et al., 2004; Mendes et al., 2009), stimulatory effects have also been reported for some species such as *Zea mays* (Danilova and Dolgikh, 2004) and *Citrus* (Oliveira et al., 2010). Stimulatory effect of antibiotics on plant morphogenesis may be explained by their chemical structure, which may mimic plant growth regulators (Nakano and Mii, 1993), or their degradation by-products, which may generate metabolites with plant growth regulator activity (Mathias and Mukasa, 1987).

Several antibiotics such as cefotaxime, augmentin, sporidex, and carbenicillin have been tested in *Jatropha* transformation procedure using different *Agrobacterium* strains (Kumar et al., 2010; Misra et al., 2012; Fu et al., 2015). Cefotaxime has been an excellent antimicrobial agent for suppressing *Agrobacterium,* especially EHA105 and LBA4404 strains (Kumar et al., 2010; Misra et al., 2012; Fu et al., 2015). Even if its inhibitory effect on shoot regeneration has been reported in some studies (Zong et al., 2010), cefotaxime, even at doses as high as 500 $mg.L^{-1}$, did not inhibit neither calluses induction nor shoots regeneration on cotyledon explants of *J. curcas* (Li et al., 2008). The complete elimination of *A. tumefaciens* EHA105 warrant transformed plant material free of bacterial contamination. In addition, cefotaxime at 500 $mg.L^{-1}$ was also stimulating the shoot regeneration on *in vitro* and *in vivo* leaf.

Considering bacterial concentration, preliminary results indicated that 0.8 OD_{600} was the most suitable density for transformation because it promoted the largest number of kanamycin-resistant shoots without having a negative impact on plant cells due to bacterial excess (data not shown). Bacterial inocula with larger OD_{600} resulted in a bacterial excess that inhibited callus induction and induced pH changes of the incubation media, which led to a corresponding reduction in transformation efficiency (Yong et al., 2006; Xu et al., 2009; Zong et al., 2010).

The *nptII* gene that confers kanamycin resistance was used here because of its recognized efficiency as a selectable marker of transformed cells in dicotyledonous plants including *J. curcas*, which have been found to be hypersensitive to kanamycin (Pan et al., 2010; Kajikawa et al., 2012). According to Tran and Mishra (2015), excessive doses of kanamycin may not only kill untransformed cells, but also inhibit the growth of transformed cells, thereby leading to lower transformation efficiency. Reduction of kanamycin concentration or/and delay selection strategies was necessary for transgenics of almond (Ramesh et al., 2006) and *J. curcas* (Fu et al., 2015). In other species such as rice (Tran and Mishra, 2015) and some trees (Stevens and Pijut, 2014), kanamycin selection is ineffective because of their natural cell resistance to this antibiotic.

In contrast to Purkayastha et al. (2010), Deng et al. (2005), Li et al. (2008) and Zong et al. (2010) who indicate low kanamycin concentration (<40 $mg.L^{-1}$) to select *J. curcas* transformed shoots, we did not find any noticeable kanamycin sensitivity of our *J. curcas* material at concentrations lower than 75 $mg.L^{-1}$ independently of explant kind (*in vitro* or *in vivo* leaf explants), which suggests that the *J. curcas* sensitivity to kanamycin is largely depending on its genotype.

Explant pre-culture has been reported to be necessary for transformation of several species with *A. tumefaciens* (Lawrence and Koundal, 2000; Barik et al., 2005; Xu et al., 2009). Kumar et al. (2010) reported that the transformation rate peaked in *J. curcas* (18.3%) when leaf explants were pre-cultured in regeneration medium for 4 days. Here, a positive effect of increasing the time of pre-culture was observed on RM until 15 days on kanamycin-resistant shoots production. The pre-culture of explants on media with phytohormones induces mesophyll cells to divide and form callus. Due to their particular dedifferentiation stage typical of highly dividing meristematic cells (Sangwan et al., 1992), these cells are competent for *Agrobacterium* transformation (Nagl et al., 1997; Guidolin, 2003; Ribas et al., 2011).

qRT-PCR was recently used to assess the copy number of transgenes due to its high sensitivity and also because of its requirement for only very small amounts of genomic DNA (Jha et al., 2011; Casu, 2012; Pinheiro et

al., 2014). Transgenic lines showed single, double and triple transgene insertions through qRT-PCR analysis and a difference of gene expression according to the internal control. Interestingly, no positive correlation has been found between copy number and expression of the trait associated to integrated gene(s) (Hobbs et al., 1990). Rather, it has been shown that a larger number of transgene copies resulted in a lower level of their expression (Beltrán et al., 2009; Hadi et al., 2012), while single or double copy integration tends to result in higher expression levels (Flavell, 1994; Pinheiro et al., 2014).

In conclusion, multiple kanamycin-resistant shoots (typically seven per explant) may be produced in 50% of explants using 15 days pre-culture on RM and selection with kanamycin (100 $mg.L^{-1}$). Based on our optimized procedure for the *J. curcas* accession considered, a *chitinase* gene was successfully introduced into an elite genotype selected for its high seed and oil yield by the IAC breeding program. Following the complete characterization of the gene insertion pattern as well as biological and field testing, transgenic genotypes will be backcrossed with other valuable genotypes to transfer them the fungal disease resistance trait. Furthermore, it was believed that we succeeded in identifying the key variables that may affect the transformation process according to the elite *J. curcas* genotype considered.

Conflict of Interests

The authors have not declared any conflict of interests.

ACKNOWLEDGEMENTS

The authors thank Petrobras for financial support and Capes (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) for Master fellowships provided to M. C. Franco and K. A. Gomes.

REFERENCES

Argollo Marques DA, Siqueira WJ, Colombo CA, Ferrari RA (2013). Breeding and Biotechnology of *Jatropha curcas*. In: Bahadur B, Sujatha M, Carels N (eds). *Jatropha*, Challenges for a New Energy Crop (V2): Genet. Improv. Biotechnol. Springer Sci. pp. 457-478.

Barik DP, Mohapatra U, Chand PK (2005). Transgenic grasspea (*Lathyrus sativus* L.): factors influencing *Agrobacterium*-mediated transformation and regeneration. Plant Cell Rep. 24:523-531.

Basa SD, Sujatha M (2009). Genetic analysis of *Jatropha* species and interspecific hybrids of *Jatropha curcas* using nuclear and organelle specific markers. Euphytica 168:197-214.

Beltrán J, Jaimes H, Echeverry M, Ladino Y, López D, Duque MC, Chavarriaga P, Tohme J (2009). Quantitative analysis of transgenes in cassava plants using real-time PCR technology. In Vitro Cell Dev Biol. Plant 45:48-56.

Bhatia P, Ashwath N, Midmore DJ (2005). Effect of genotype, explant orintation, and wounding on shoot regeneration in tomato. *In vitro* Cellular Dev. Biol. Plant. 41:441-457.

Bookout AL, Mangelsdorf DJ (2003). Quantitative real-time PCR protocol for analysis of nuclear receptor signaling pathways. Nucl. Recept. Signal. 1 e 012.

Carels N (2013). Towards the domestications of *Jatropha*: The integration of sciences. *In:* Bahadur B, Sujatha M, Carels N (eds). *Jatropha*, Challenges for a New Energy Crop (V2): Genet. Improv. Biotechnol. Springer Sci. pp. 263-299.

Casu RE, Selivanova A, Perroux JM (2012). High-throughput assessment of transgene copy number in sugarcane using real-time quantitative PCR. Plant Cell Rep. 31:67-177.

Debnath M, Bisen PS (2008). *Jatropha curcas* L., a multipurpose stress resistant plant with a potential for ethnomedicine and renewable energy. Curr. Pharm. Biotechnol. 9(4):288-306.

Danilova SA, Dolgikh YI (2004). The stimulatory effect of the antibiotic cefotaxime on plant regeneration in maize tissue culture. Russ. J. Plant Physiol. 51:621-625.

Deng JP, Yan SH, Hou P, Xu Y, Wang S, Tang L, Yan F, Chen F (2005). Effects of different antibiotics at various concentrations on culture of *Jatropha curcas*. Chin. J. Appl. Environ. Biol. 11:156-159.

Deore AC, Johnson TS (2008). High-frequency plant regeneration from leaf-disc cultures of *Jatropha curcas* L.: an important biodiesel plant. Plant Biotechnol. Rep. 2:7-11.

Divakara BN, Upadhyaya HD, Wani SP, Laxmipathi Gowda CLL (2010). Biology and genetic improvement of *Jatropha curcas* L.: a review. Appl. Energ. 87:732-742.

Doyle JJ, Doyle JL (1990). Isolation of plant DNA from fresh tissue. Focus. 12:13-15.

Flavell RB (1994). Inactivation of gene expression in plants as a consequence of specific sequence duplication. Proc. Natl. Acad. Sci. USA 91:3490–3496.

Franco MC, Latado RR, Siqueira WJ, Marques DA (2014). Micropropagation of *Jatropha curcas* superior genotypes and evaluation of clonal fidelity by target region amplification polymorphism (TRAP) molecular marker and flow cytometry. Afr. J. Biotechnol. 13:3872-3880.

Fu Q, Li C, Tang M, Tang M, Tao YB, Pan BZ, Zang L, Niu L, He, H, Wang X, Xu ZF (2015). An efficient protocol for *Agrobacterium*-mediated transformation for the biofuel plant *Jatropha curcas* by optimizing kanamycin concentration and duration of delayed selection. Plant Biotechnol. Rep. 9(6):405-416.

Giersdorf J (2013). Politics and economics of ethanol and biodiesel production and consumption in Brazil. Accessed August 2016: https://www.dbfz.de/fileadmin/user_upload/DBFZ_Reports/DBFZ_Report_15.pdf.

Guidolin AF (2003). Regeneração de plantas de *Phaseolus vulgaris* L. a partir de calos e transformação genética via *Agrobacterium.* 100f. Dissertação (Doutorado em Ciências) - Universidade de São Paulo, Piracicaba. Portuguese.

Hadi F, Salmanian AH, Ghazizadeh E, Amani J, Noghabi KA, Mousavi A (2012). Development of quantitative competitive PCR for determination of copy number and expression level of the synthetic glyphosate oxidoreductase gene in transgenic canola plants. Electron. J. Biotechnol. 15(4):2-2.

Herr J, Carlson J (2013). Traditional breeding, genomics-assisted breeding, and biotechnological modification of forest trees and short rotation woody crops. In: Jacobson M, Ciolkosz D, editors. Wood-based Energy in the Northern Forests. New York: Springer; pp. 79-99.

Hobbs SLA, Kpodar P, DeLong CMO (1990). The effect of T-DNA copy number, position and methylation on reporter gene expression in tobacco transformants. Plant Mol. Biol. 15(6):851-864.

Jha TB, Mukherjee P, Datta MM (2007). Somatic embryogenesis in *Jatropha curcas* Linn., an important biofuel plant. Plant Biotechnol. Rep. 1:135-140.

Jha B, Sharma A, Mishra A (2011). Expression of SbGSTU (Tau class glutathione S-transferase) gene isolated from *Salicornia brachiata* in tobacco for salt tolerance. Mol. Biol. Rep. 38:4823-4832.

Jha B, Mishra A, Jha A, Joshi M (2013). Developing transgenic *Jatropha* using the *SbNHX1* gene from an extreme halophyte for cultivation in saline wasteland. PLoS ONE. 8:8.

Joshi M, Mishra A, Jha B (2011). Efficient genetic transformation of *Jatropha curcas* L. by microprojectile bombardment using embryo axes. Ind. Crop. Prod. 33:67-77.

Kajikawa M, Morikawa K, Inoue M, Widyastuti U, Suharsono S, Yokota A, Akashi K (2012). Establishment of bispyribac selection protocols for *Agrobacterium tumefaciens*-and *Agrobacterium rhizogenes*-mediated transformation of the oil seed plant *Jatropha curcas* L. Plant Biotechnol. 29:145-153.

Kaushik N, Kumar K, Kumar S (2007). Genetic variability and divergence studies in seed traits and oil content of Jatropha (*Jatropha curcas* L.) accessions. Biomass Bioenerg. 31:497-502.

Kumar N, Reddy MP (2010). Plant regeneration through the direct induction of shoot buds from petiole explants of *Jatropha curcas*: a biofuel plant. Ann. Appl. Biol. 156:367-375.

Kumar N, Reddy MP, Sujatha M (2013). Genetic transformation of *Jatropha curcas:* Current status and future prospects. *In:* Bahadur B, Sujatha M, Carels N (eds). *Jatropha*, Challenges for a New Energy Crop (V2):): Genet. Improv. Biotechnol. Springer Sci. Pp. 457-478.

Kumar N, Vijay Anand KG, Sudheer Pamidimarri DVN, Sarkar T, Reddy MP, Radhakrishnan T, Kaul T, Reddy MK, Sopori SK (2010). Stable genetic transformation of *Jatropha curcas* via *Agrobacterium tumefaciens*-mediated gene transfer using leaf explants. Ind. Crops Prod. 32: 41-47.

Kumar N, Vijayanand KG, Reddy MP (2011). Plant regeneration of non-toxic *Jatropha curcas*—impacts of plant growth regulators, source and type of explants. J. Plant Biochem. Biotechnol. 20: 125-133.

Lawrence PK, Koundal KR (2000). Simple protocol for *Agrobacterium tumefaciens* - mediated transformation of pigeonpea [*Cajanus cajan* (L.) Millsp.]. J. Plant Biol. 27(3):299-302.

Li MR, Li HQ, Jiang HW, Pan XP, Wu JG (2008). Establishment of an *Agrobacterium*-mediated cotyledon disc transformation method for *Jatropha curcas*. Plant Cell Tiss. Organ Cult. 92:173-181.

Machado AA, Zonta EP (1995). Manual do SANEST: Sistema para análise estatística para microcomputadores. Pelotas, UFPel. 102.

Mason G, Provero P, Vaira AM, Acotto GP (2002). Estimating the number of integrations in transformed plants by quantitative real-time PCR. BMC Biotechnol. 2:20-30.

Mathias RJ, Mukasa C (1987). The effect of cefotaxime on the growth and regeneration of callus from four varieties of barley (*Hordeum vulgaris* L.). Plant Cell Rep. 6:454-557.

da Silva Mendes AF, Cidade LC, de Oliveira MLP, Otoni WC, Soares-Filho WS, Costa MGC (2009). Evaluation of novel beta-lactam antibiotics in comparison to cefotaxime on plant regeneration of *Citrus sinensis* L. Osb. Plant Cell Tiss. Organ Cult. 97:331-336.

Miller JH (1972). Experiments in Molecular Genetics. Cold Spring Harbor Laboratory, New York 295 p.

Misra P, Toppo DD, Mishra MK, Saema S, Singh G (2012). *Agrobacterium tumefaciens*-mediated transformation protocol of *Jatropha curcas* L. using leaf and hypocotyl segments. J. Plant Biochem. Biotechnol. 21:128-133.

Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tabacco tissue cultures. Physiol. Plant. 15:473-497.

Nagl W, Ignacimuthu S, Becker J (1997). Genetic engineering and regeneration of *Phaseolus* and *Vigna*. State of the art and new attempts. J. Plant Physiol. 150:625-644.

Nanasato Y, Kido M, Kato A, Ueda T, Suharsono, Widyastuti U, Tsujimoto H, Akashi K (2015). Efficient genetic transformation of *Jatropha curcas* L. by means of vacuum infiltration combined with filter-paper wicks. *In vitro* Cell. Dev. Biol. Plant 51:399-406.

Nakano M, Mii M (1993). Antibiotics stimulate somatic embryogenesis without plant growth regulators in several *Dianthus* cultivars. J. Plant Pathol. 141:721-725.

Oliveira MLP, Costa MGC, Silva CV, Otoni WC (2010). Growth regulators, culture media and antibiotics in the *in vitro* shoot regeneration from mature tissue of citrus cultivars. Pesq. Agropec. Bras. 45(7):654-660.

Openshaw K (2000). A review of *Jatropha curcas*: an oil plant unfulfilled promise. Biomass Bioeng. 19:1-15.

Pan J, Fu Q, Xu ZF (2010). *Agrobacterium tumefaciens*-mediated transformation of biofuel plant *Jatropha curcas* using kanamycin selection. Afr. J. Biotechnol. 9:6477-6481.

Pinheiro TT, Figueira A, Latado RR (2014). Early-flowering sweet orange mutant “x11” as a model for functional genomic studies of Citrus. BMC Res. Notes 7: 511.

Purkayastha J, Sugla T, Paul A, Solleti SK, Mazumdar P, Basu A, Mohommad A, Ahmed Z, Sahoo L (2010). Efficient *in vitro* plant regeneration from shoot apices and gene transfer by particle bombardment in *Jatropha curcas.* Biol. Plant. 54:13-20.

Rao GR, Korwar GR, Shanker AK, Ramakrishna YS (2008). Genetic associations, variability and diversity in seed characters, growth, reproductive phenology and yield in *Jatropha curcas* (L.) accessions. Trees 22(5):697-709.

Ramesh SA, Kaiser BN, Franks T, Collins G, Sedgley M (2006) Improved methods in *Agrobacterium*-mediated transformation of almond using positive (mannose/pmi) or negative (kanamycin resistance) selection-based protocols. Plant Cell Rep. 25:821-828.

Ribas AF, Dechamp E, Champion A, Bertrand B, Combes MC, Verdeil JC, Lapeyre F, Lashermes P, Etienne H (2011). *Agrobacterium*-mediated genetic transformation of *Coffea arabica* (L.) is greatly enhanced by using established embryogenic callus cultures. BMC Plant Biol. 11:92.

Rozen S, Skaletsky H (2000). Primer3 on the WWW for general users and for biologist programmers. In Bioinformatics methods and protocols: methods in molecular biology. S. Misener and S.A. Krawetz, editors. Humana Press. Totowa, New Jersey, USA, pp. 365-386.

Sabandar CW, Ahmat N, Jaafar MJ, Sahidin L (2013). Medicinal property, phytochemistry and pharmacology of several *Jatropha* species (Euphorbiaceae): A review. Phytochem. 85:7-29.

Sangwan RS, Bourgeoiis Y, Brown S, Vasseur G, Sangwan-Norreel B. (1992). Characterization of competent cells and early events of *Agrobacterium*-mediated genetic transformation in *Arabidopsis thaliana*. Plant. 188:439-456.

Sharma S, Kumar N, Reddy MP (2011). Regeneration in *Jatropha curcas*: factors affecting the efficiency of *in vitro* regeneration. Ind. Crop Prod. 34:943-951.

Stevens ME, Pijut, PM (2014). *Agrobacterium*-mediated genetic transformation and plant regeneration of the hardwood tree species *Fraxinus profunda*. Plant Cell Rep. 33:861-870.

Sujatha M, Bahadur B, Reddy TP (2013). Interspecific hybridization in the genus *Jatropha*. In: Bahadur B, Sujatha M, Carels N (eds). *Jatropha*, Challenges for a New Energy Crop (V2): Genet. Improv. Biotechnol. Springer Sci. pp. 423-443.

Tang W, Luo H, Newton RJ (2004). Effects of antibiotics on the elimination of *Agrobacterium tumefaciens* from loblolly pine (*Pinus taeda*) zygotic embryo explants and on transgenic plant regeneration. Plant Cell Tiss. Organ Cult. 79:71-81.

Toppo DD, Singh G, Purshottam D, Misra P (2012). Improved *in vitro* rooting and acclimatization of *Jatropha curcas* plantlets. Biomass Bioenerg. 44:42-46.

Tran TN, Mishra, NS (2015). Effect of antibiotics on callus regeneration during transformation of IR 64 rice. Biotechnol. Rep. 7:143-149.

Varshney A, Johnson TS (2010). Efficient plant regeneration from immature embryo cultures of *Jatropha curcas*, a biodiesel plant. Plant Biotechnol. Rep. 4(2):139-148.

Visarada K, Meena K, Aruna C, Srujana S, Saikishore N, Seetharama N (2009). Transgenic breeding: perspectives and prospects. Crop Sci. 49:1555-1563.

Wani SP, Osman M, D'silva E, Sreedevi TK (2006). Improved livelihoods and environmental protection through biodiesel plantations in Asia. Asian Biotechnol. Develop. Rev. 8(2):11-29.

Wei Q, Lu WD, Liao Y, Pan SL, Xu Y, Tang L, Chen F (2004). Plant regeneration from epicotyl explant of *Jatropha curcas*. J. Plant Physiol. Mol. Biol. 30:475-478.

Xu J, Wang YZ, Xia Yin H, Jing Liu X (2009). Efficient *Agrobacterium tumefaciens*-mediated transformation of *Malus zumi* (Matsumura) Rehd using leaf explant regeneration system. Electron. J. Biotechnol. 12(1):3-4.

Yong WTL, Abdullah JO, Mahmood M (2006). Optimization of *Agrobacterium* mediated transformation parameters for

Melastomataceae spp. using green fluorescent protein (GFP) as a reporter. Sci. Hortic. 109:78-85.
Yu TA, Yeh SD, Yang JS (2001). Effects of carbenicillin and cefotaxime on callus growth and somatic embryogenesis from adventitious roots of papaya. Bot. Bull. Acad. Sin. 42:281-286.
Zong H, Wang S, Ouyang C, Deng X, Li L, Li J, Chen F (2010). *Agrobacterium*-mediated transformation of *Jatropha curcas* young leaf explants with lateral shoot-inducing factor (LIF). Int. J. Agric. Biol. 12:891-896.