UNIVERSIDADE FEDERAL DO PARANÁ

GISELA MANUELA DE FRANÇA BETTENCOURT

INDIRECT ORGANOGENESIS AND GENETIC TRANSFORMATION PROTOCOL DEVELOPMENT FOR AN ELITE CLONE OF *E. uropylla*

CURITIBA

2016

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Dissertation submitted to the Post-Graduation Program in Bioprocess and Biotechnology Engineering, concentration area of Agroindustrial Biotechnology, Agricultural Sciences Sector, Federal University of Parana, as part of the requirements to obtain a Master's degree in Bioprocess and Biotechnology Engineering.

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CURITIBA

2016

B565i

Bettencourt, Gisela Manuela de França
Indirect organogenesis and genetic transformation protocol development for an elite clone of E. uropylla/ Gisela Manuela de França Bettencourt. – Curitiba, 2016.
105 f. : il. color. ; 30 cm.

Dissertação - Universidade Federal do Paraná, Setor de Tecnologia, Programa de Pós-graduação em Engenharia de Bioprocessos e Biotecnologia, 2016.

Orientador: Carlos Ricardo Soccol – Co-orientador: Juliana Degenhardt-Goldbach.
Bibliografia: p. 98-103.

1. Agrobacterium tumefaciens. 2. Oraganogênese. 3. Enzimas - Biotecnologia. 4. Pseudomonas beteli. I. Universidade Federal do Paraná. II.Soccol, Carlos Ricardo. III. Degenhardt-Goldbach, Juliana . IV. Título.

CDD: 583.765135

TERMO DE APROVAÇÃO

GISELA MANUELA DE FRANCA BETTENCOURT

"INDIRECT ORGANOGENESIS AND GENETIC TRANSFORMATION PROTOCOL DEVELOPMENT FOR AN ELITE CLONE OF *E. uropylla*"

Dissertação aprovada como requisito parcial para obtenção do grau de Mestre no Programa de Pós-Graduação em Engenharia de Bioprocessos e Biotecnologia, Setor de Tecnologia, Universidade Federal do Paraná, pela seguinte banca examinadora:

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Curitiba, 23 de Fevereiro de 2016.

I dedicate this work to my parents,
João and Nela, and to my beloved brother
Joel Bettencourt, for all the effort and
Love to make this dream come true.

ACKNOWLEDGMENT

First I would thank GOD for all blessings and let me live to concretize my dreams.

I would like to thank Dr. Carlos Ricardo Soccol for the opportunity and confidence in this research. Thanks to Dr[a] Juliana Degenhardt-Goldbach for the orientation, to cheer her knowledge, for the moments of fellowship and friendship over the years.

I also would like to sincerely thank the Paraná Federal University, Post-Graduation of Bioprocess and Biotecnology Engeneering Program by enabling the realization of my master, to Embrapa Foresty for providing me assistantship to support my research and also to CAPES for the financial support.

A special acknowledgement to my parents, my pillars that always helped me to realize my dreams, thanks for the love, affection and trust. My brothers for the affection and love, in special to Joel, may your soul rest in peace. Yannick my cousin by the company. My sincere thanks to Marco for the moments of companionship, affection and for the time to be present.

Thanks are extendent to all my friends. For the girls from Plant Tissue Culture Lab and Molecular Biology Laboratory: Janaina, Cassiana, Renata, Jessica, Thais, Laudiane, Fabricina, Mariane and Gheniffer, for the companionship and friendship, the laughter and moments of relaxation, the aid even if expressed in motivational words. To Daiane and Germana who also contributed to accomplishment of the work, with their wisdom. To my friends Jaqueline, Hagira, Atifa, Auria and Júlia, even far away, always were present for me. Thank you for the moments of affection and companionship, you are like sisters to me. Thanks to my colleagues from Rotaract and Rotary. My rotarians family, thank you. Thanks for the colleagues from the Bioprocess and Biotechnology Engineering Program.

And I also would like to thank the defense of banking, composed by Dr. Carlos Ricardo Soccol, Dra. Juliana Degenhardt-Goldbach, Dr. André Luis Lopes da Silva e Dra. Giovana Bomfim de Alcantara, to dispose of their time for consideration of this work.

And to all who somehow twisted and contributed to the realization of this dream, thank you very much.

Success is obtained only by increasing your strengths,

not by the elimination of old weakness.”

Author unknown

RESUMO

Este trabalho teve por objetivos desenvolver um protocolo de regeneração por organogênese indireta e um protocolo de transformação genética para o clone BRS07-01 de *E. urophylla,* além de identificar e caraterizar uma bactéria endofítica isolada deste clone e avaliar sua interação com a *Agrobacterium tumefaciens.* Com relação a organogênese, a melhor taxa de regeneração (85.6%) foi observada quando os explantes foram primeiramente cultivados em meio de indução de calos WPM suplementado com 0.5 µM de TDZ e 0.5 µM de ANA e em seguida transferidos para o meio de indução de brotos com 5.0 µM de BAP e 1.0 µM de ANA. Após a regeneração dos brotos, a melhor taxa de enraizamento (84.0%) foi obtida em meio suplementado com 14.7 µM de IBA. Com base na avaliação de 56 marcadores RAPD não foi observada variação somaclonal durante as fases da organogênese. Dois agentes seletivos foram testados para aumentar a eficiência de seleção, canamicina e geneticina, e a concentração mínima de 50 mgL^{-1} de canamicina foi selecionada para a transformação genética. Para o desenvolvimento de protocolo de transformação genética, vários fatores foram avaliados como dias de pré e co-cultivo, presença de Acetosyringona (AS), concentrações de agentes seletivos, tipo de explantes e tempo de sonificação. Quando usado o explante foliar, a maior eficiência de transformação genética (TE) foi de 2.67%, utilizando 50 µM de AS no co-cultivo líquido e 100 µM de AS no co-cultivo sólido. Entretanto, a maior TE de 20.83% foi observada com brotações como explantes submetidos a 2 min de sonificação e co-cultivados com 100 µM de AS e selecionados em meio com 150 mgL^{-1} de Canamicina. Durante os experimentos, observamos a presença de bactérias endofíticas. Após isoladas, com base na análise filogenética, dois isolados foram identificados como *Stenotrophomonas maltophilia*, uma bactéria gram-negativa com sensibilidade a diferentes antibiótiocos, capaz de formar biofilme e sintetizar a auxina ácido indol-acético, que pode estar interferindo no processo e nos resultados de transformação genética.

Palavras-chave: *Agrobacterium tumefaciens*, organogênese indireta, acetosseringona, *GUS*, *nptII*, *Stenotrophomonas maltophilia*

ABSTRACT

This work aimed to develop an indirect organogenesis and a genetic transformation protocol for clone BRS07-01 of *E. urophylla,* as well as to isolate and characterize endophytic bacteria from this clone, which could be interfering in the *Agrobacterium tumefaciens* infection process during transformation of the clone. Regarding the organogenesis, the results showed that higher regeneration rate (85.6%) was observed when the leaves explants were first cultured on callus induction medium WPM supplemented with 0.5 µM of TDZ and 0.5 µM of NAA and then transferred to the shot induction medium with 5.0 µM of BAP and 1.0 µM NAA. The best adventitious root formation (80.0%) was observed when plantlets were cultured on medium supplemented with 14.7 µM IBA. Based on 56 RAPD molecular markers we did not observe somaclonal variation during the organogenesis process. Two selective agents, kanamycin and geneticin, were tested to determine the best antibiotic and concentration for genetic transformation. Kanamycin at 50 mgL^{-1} showed to be the most effective. For genetic transformation, several factors were evaluated, as days on pre and co-culture, Acetosyringone (AS), concentrations of selective agents, explants type and sonication time. For leaf explants, higher transformation efficiency (TE) was 2.67% when the medium was supplemented with 50 µM of AS on liquid co-culture and 100 µM of AS on solid co-culture. However, the highest TE (20.83%) was observed when with microshoots were used as explants and they were submitted to 2 min of sonication, co-cultured with 100 µM of AS and selected on medium with 150 mgL^{-1} of Kanamycin. Two isolates endophytic bacteria could be isolated and characterized as *Stenotrophomonas maltophilia*. This gram-negative bacteria, showed sensibility to different antibiotics and was able to form biofilm and synthetize the auxin indolacetic acid. For these reasons, we suggest that this endophytic can somehow be interfering in the genetic transformation of the BRS07-01 clone.

Index terms: *Agrobacterium tumefaciens*, indirect organogenesis, Acetosyringone, *GUS*, *nptII*, *Stenotrophomonas maltophilia*

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LIST OF ABBREVIATIONS

2,4-D – diclorofenoxiacetic acid

µM – micro Molar

ACC - 1-aminocyclopropane-1-carboxylate

AS- Acetosyringone

BAP – Benzilaminepurine

CIA - chloroform:isoamyl alchol

CIM – callus induction media

CTAB - Cetyltrimethylammonium bromide

IAA – indolacetic acid

IBA- indolbutiric acid

FAA - formaldehyd/ethanol/acetic acid

GUS - beta-glucuronidase

Gen- Geneticin

LB - Lysogeny broth

Km- Kanamycin

MS - Murashige and Skoog medium

NAA - 1-Naphthaleneacetic acid

OD_{600} – Optic dendity

PVP – polyvinylpyrrolidone

PGRs – Plant Growth regulators

RAPD – Rapid Amplified Polymorphic DNA

RPM – Rotation per minute

TDZ – Thidiazuron

TE- transformation efficiency

SAAT- sonication-assisted *Agrobacterium*-mediated transformation

SE- selective efficiency

SIM – shoots induction media

WPM – Wood Plant medium

X-gluc - 5-bromo-4-chloro-3-indolyl-beta-D-glucuronic acid

SUMMARY

INTRODUCTION

Planted tree industry in Brazil represents 91% of all the wood resource for the industry. This sector plays an important role in providing environmental services and represents one of the most productive sectors in the Brazilian economy as demonstrated by the last commercial balance from 2014, with an amount of US$ 924 Million FOB from Pulp and Paper industry.

Eucalyptus from the Myrtaceae family, is the major genus planted in Brazil, reaching a productivity over 60 m^3/ha in 7 years rotation. These species represents 71.9% of the total forestry planted area, covering over 5.56 million ha and leads Brazil to the largest eucalyptus pulp producer worldwide. The high adaptability and phenotypic plasticity within its 700 known species are among the most interesting characteristics of this genus. *Eucalyptus urophylla,* commonly found in tropical regions, is one of the most planted species in Brazil due its higher productivity and potentiality to grow in diverse regions and also due its tolerance to cancer attack of *Cryphonectria cubensis*.

Even though the forestry sector is very lucrative, the world challenge now is to meet the demands for food and natural resources for the next decades. The United Nations foresees a global population of 9.5 billion by 2050, which will require a 70% increase of worldwide food production and increase of 40% of wood for industrial purposes and energy generation demand. This scenario will take us to expand billions of hectares of land for agriculture crops and trees plantation. So, it will directly compete with infrastructure networks and urban areas expansion, and also will need to face the climate challenges, which day by day are becoming more unpredictable and devastating.

In this context, governments and private companies seek for technological innovation by combining conventional genetic breeding techniques and biotechnology to overcome these challenges. In the forestry sector, biotechnology techniques, as tissue culture, were well adapted to promote clonal micropropagation system and cryopreservation, and genetic engineering emerged as a powerful tool to molecular breeders to increase genetically improved material for plantations, and for a deeper understanding of the genes controlling commercially important traits.

Despite progress in recent decades, efforts still must be done in forest sector in respect of genetic improvement. This sector stills so far away from those achieved for crops, even that various research groups are involved and reported different genetic transformation protocols and attempts towards development of transgenic trees.

The major traits of interest in *Eucalyptus* genetic enhancement via genetic transformation have been mainly focused on cellulose modification or biosynthesis, biomass increase, lignin and cellulose content modification, tolerance to biotic and abiotic stresses, phytoremediation enhancement, sterility and root ability. Most of the published reports are related to genetic transformation protocol development and improvement.

Notwithstanding, some advances have been occurring in respect of *Eucalyptus* genetic transformation. From 2002 to 2011, a tree biotechnology company, ArborGen, conducted field testes of genetically modified *Eucalyptus* tolerant to freeze stress in Southern USA to provide an economically viable hardwood option in the region (authorized by APHIS - Animal and Plant Health Inspection Service). And recently another Biotechnology company, Futuragene gained the liberation to cultivate in the field a transgenic *E. grandis* x *E. urophylla* hybrid, which showed a high volumetric wood increase in Brazil.

Nevertheless, the low genetic transformation efficiency is still the main stumbling block for *Eucalyptus* species, which is mainly pointed to the recalcitrance of *Eucalyptus* species. However, to achieve high efficiency on genetic transformation, its necessary to evaluate all the factors that can influence the efficiency. And one of the most important points is the regeneration method, which will allow the growth and recovery of the transformed explants. Normally, the regeneration method its done by direct or indirect organogenesis, where an organized integrated mass of cells passes through dedifferentiation process to form a complete new tissue/organ. Nonetheless, it is important to develop and optimize a successful regeneration protocol for each plant species or even clone with which it is proposed to work with. Other aspect which also may affect the genetic transformation is the nature of the explants and genotype susceptibility to *Agrobacterium* strains.

Microorganism infections are a serious problem for *in vitro* tissue culture. The environmental its appropriate for a microorganism contamination or even to accentuate presence of an existing endophytic microorganism. We could observe

high interference of an endophytic bacterium while we were performing our genetic transformation experiments. The presence of this endophytic it seemed to be interfering on the plant growth or even in genetic transformation process, interfering the *Agrobacterium* infection.

GENERAL OBJETIVE

To obtain highly efficient organogenesis and genetic transformation protocols for clone BRS07-01 of *Eucalyptus urophylla* and to isolate and characterize endophytic bacteria isolated from this clone.

SPECIFIC OBJECTIVES

1. To evaluate different concentrations and combinations of citokynins and auxins in the callus induction medium for organogenesis of the clone BRS07-01.
2. To evaluate different concentrations of BAP and NAA on shoot induction medium to promote shoot formation in the organogenesis protocol of the clone.
3. To evaluate the best combination of the callus induction medium and the shoot induction medium to achieve the higher regeneration rate on the organogenesis of the clone.
4. To evaluate the effect of IBA on root formation of the *in vitro* plantlets of the clone.
5. To evaluate somaclonal variation among the different *in vitro* stages of organogenesis using RAPD molecular markers.
6. To analyze the effects of the selective agents Kanamycin and Geneticin on the regeneration of leaves of the clone.
7. To evaluate different factors during genetic transformation of clone BRS07-01 via *Agrobacterium tumefaciens*: pre and co-culture days, Acetosyringone, concentrations of Kanamycin and Geneticin, type of explants and sonication time.

8. To identify and characterize endophytic bacteria isolated from clone BRS07-01 and to evaluate their possible interference in the *Agrobacterium*-mediated transformation of clone BRS07-01.

CHAPTER I: High efficiency organogenesis and analysis of genetic stability in an elite clone of *Eucalyptus urophylla*

ABSTRACT

Genetic transformation is becoming routine for engineering stress resistance in recalcitrant species such as *Eucalyptus*. Even though, regeneration protocols are still bottlenecks in the protocols of transgenic plants. In functional genomics, desired genes are initially cloned in model plants such as tobacco or poplar before transformation into *Eucalyptus*. However, the genetic distance between the model and target species can influence the results. Therefore, developing a *Eucalyptus* clone with high organogenetic potential could be of interest as a model plant for such studies. This work aimed to develop a high efficiency *in vitro* organogenesis protocol for the clone BRS07-01 of *E. urophylla* in order to improve its genetic transformation. We also assessed genetic stability during the *in vitro* culture stages. Plant growth regulators were evaluated for indirect organogenesis and rooting. Transgenic plants expressing the *gus* gene were obtained via *Agrobacterium tumefaciens* and selected for kanamycin resistance. A RAPD analysis was performed to check for somaclonal variation. In a two-step protocol, the combination of callus induction media supplemented with 0.5 µM TDZ + 0.5 µM NAA and shoot induction media supplemented with 5.0 µM BAP + 1.0 µM NAA allowed up to 85.6% shoot formation. It also increased the number of shoots per explants. On rooting medium supplemented with 4.9 µM IBA, we observed 22.5% explants forming roots and over 80% were successfully acclimatized. The clone was successfully transformed with the *gus* gene under the 35S promoter. By testing eleven RAPD primers we did not observe somaclonal variation on the in *vitro* stages evaluated.

Keywords: *in vitro* regeneration, genetic transformation, *Agrobacterium tumefaciens*, model plant, RAPD

1. INTRODUCTION

Since the discovery of the totipotence and plant growth regulators (PGRs), tissue culture has been widely used to overcome bottlenecks or time consuming procedures in breeding programs and vegetative propagation techniques. In vitro culture is used to provide large scale clonal propagation, improve rooting, promote rejuvenation of selected clones, produce commercially valuable secondary metabolites, and as an essential tool for genetic engineering (ALCANTARA *et al.*, 2011; BRONDANI *et al.*, 2012; EZHOVA, 2003; ELISSETCHE *et al.*, 2011; MENDONÇA *et al.*, 2013).

The genomics era has enabled sequencing of specific genes and in some cases full genomes. However, gene sequencing alone does not disclose which genes are transcriptionally active in different metabolic pathways, tissues or developmental stages (BRITT and MAY, 2003). More recently, the field of functional genomics has enabled elucidation of gene functions, which has made a tremendous contribution to plant breeding (BARONE *et al*., 2008; MITSUDA and OHME-TAKAGI, 2009). One way to validate the sequenced genes through functional genomics is through genetic transformation of model plants.

Genetic transformation of model plants is a very efficient method for the validation of gene function. Through this, plants are transformed with the gene to be studied, so that overexpress or silence expression. Plants are then evaluated phenotypic, biochemical and physiological and compared with wild type plants to determine in which metabolic pathways the gene are involved (TAYLOR, 2002). Some species are established as model plants such as *Arabidopsis thaliana* (MEINKE *et al.*, 1998) and rice (SHIMAMOTO and KYOZUKA, 2002). However, the evolutionary distance with respect to perennials can be considerable, especially when the goal is the study of characteristics directly linked to tree species. Currently, only selected *Populus* species are used as model plants for perennial species. But the genetic distance remains a challenge in using *Populus* species for transformation and regeneration protocols on other perennials such as *Eucalyptus*.

However, if we were able to find a model plant in the same genus, from which the genes are isolated, validation would be easier to be transferred, since it has been observed in some studies, that expression in the plant model differed from that observed in the species from which the gene was isolated (BRANDALISE, 2007;

COSTA, 2011; RIBEIRO, 2009; RODRIGUES *et al.*, 2013; VAUGHAN *et al.*, 2006). Therefore, if we could develop a highly efficient protocol for a *Eucalyptus* clone, it could be used as a model plant for gene validation studies.

Once genotype has a high influence on regeneration, it is important to develop and optimize a successful regeneration protocol for each plant species or even clone with which it is proposed to work with. The regeneration can be achieved by direct or indirect organogenesis, a complex process which relies on induction of meristematic activity in mature differentiated cells (GEORGE *et al.*, 2008).

Eucalyptus is one of the main forest genera planted globally because of its adaptability, rapid growth, high-quality wood products and low cost of wood pulp fibers (MENDONÇA *et al.*, 2013). As nowadays *Eucalyptus* represent 71.9% of planted forests in Brazil with over 5 million ha (IBÁ, 2015).

In vitro organogenesis of *Eucalyptus* species has been frequently reported: *E. grandis* (HAJARI *et al.*, 2006), *E. urophylla* (TIBOK *et al.*, 1995), and *E. grandis* x *E. urophylla* (ALCANTARA *et al.* 2011; ALVES *et al.* 2004). However, *Eucalyptus* species tend to be recalcitrant, limiting the capacity of *in vitro* regeneration (MYCOCK and WATT, 2012; QUOIRIN and QUISEN, 2006; TIBOK *et al.*, 1995; TOURNIER *et al.*, 2003). We therefore set out to optimize explant regeneration of BRS07-01 clone of *E. urophylla*. This clone was chosen once it is one of the most promising materials of the *Eucalyptus* breeding program of Embrapa Forestry. It possesses characteristically fast growth (wood volume of 0,047 m^3/tree at the 21^{st} month) and is suitable for multiple uses (Estefano Paludzyszyn Filho, Embrapa Forestry researcher, personal communication).

Mutations and somaclonal variations can occur during tissue culture and may later compromise field performance (AGGARWAL *et al.*, 2010). This can be quite costly for large commercial operations (MIÑANO *et al.*, 2009; WOLFF and PETERS-VAN RIJN, 1993). Thus after optimizing our regeneration protocol, we screened our explants for somaclonal variation using the Random Amplified Polymorphic DNA (RAPD) technique.

This study aimed to: (i) develop an efficient two-step *in vitro* organogenesis protocol for of *E. urophylla* BRS07-01 and (ii) to evaluate somaclonal variation among the different *in vitro* stages of regeneration.

2. MATERIAL AND METHODS

2.1. Plant material and culture conditions

As explants we used leaves from shoots of in vitro grown plants of *E. urophylla* BRS07-01 clone maintained on MS media (MURASHIGE and SKOOG, 1962) supplemented with 30 gL^{-1} sucrose, 0.88 µM BAP (6-benzylaminopurine), 0.2 mgL^{-1} myo-inositol and 7 gL^{-1} agar at Embrapa Forestry, Colombo- Brazil. The pH of all media was adjusted to 5.8 before autoclaving for 20 min at 120°C 1 atm. All the cultures were maintained at 23 ±2°C in dark or 16h photoperiod, as described.

2.2. In vitro indirect organogenesis

The basal medium for indirect organogenesis was based on those described by Oliveira et al. (2015). Leaves were inoculated, with the adaxial side in contact with the media, on petri dishes with 20 mL of callus induction medium (CIM) WPM (LLOYD and MCCOWN, 1981) containing 30 gL^{-1} sucrose, 0.5 µM TDZ (Thidiazuron), 0.1 µM NAA (1-Naphthaleneacetic acid), 0.1 mgL^{-1} myo-inositol, 500 mgL^{-1} PVP and 7.0 gL^{-1} agar and incubated for 30 days at 23 ±2°C in dark. Explants were then transferred to shooting induction medium (SIM) containing WPM salts and vitamins, 30 gL^{-1} sucrose, 5.0 µM BAP, 0.5 µM NAA, 0.1 mgL^{-1} myo-inositol, 500 mgL^{-1} PVP and 7.0 gL^{-1} agar, and incubated for 60 days in a growth room on 16h of photoperiod.

2.3. Effect of Plant growth regulators on indirect organogenesis (CIM)

In this experiment, different PGRs were tested on CIM medium to evaluate the most efficient combination and concentration to induce callus formation and shoot proliferation. We tested the cytokinins TDZ and BAP, and auxins NAA and 2,4-D (2,4-dichlorophenoxyacetic acid) in the following treatments (in µM) : T1: 0.25 TDZ + 0.1 NAA; T2: 0.5 TDZ + 0.1 NAA; T3: 0.75 TDZ + 0.1 NAA; T4: 1.0 TDZ + 0.1 NAA; T5: 2.0 TDZ + 0.1 NAA; T6: 0.5 TDZ + 0.2 NAA; T7: 0.5 TDZ + 0.5 NAA; T8: 0.5 TDZ + 0.1 2,4D; T9: 0.5 TDZ + 0.2 2,4-D; T10: 5.0 BAP + 0.1 NAA; T11: 5 BAP + 0.5 NAA; T12: 5 BAP + 1.0 NAA; T13: 5 BAP + 2.5 NAA; T14: 7,5 BAP + 0.5 NAA; T15: 2.5 BAP + 0.5 NAA; T16: 7.5 BAP + 0.1 NAA; T17: 0.25 TDZ + 2.5 BAP. After 30 days, all the explants were transferred to the basal SIM.

2.4. Effect of BAP and NAA on shoot induction medium (SIM)

To optimize the shoot induction medium, five concentrations and combinations of BAP and NAA (in µM) were tested: T1: 5 + 0.1; T2: 5 + 0.5; T3: 5 + 1; T4: 2.5 + 0.5; T5: 7.5 + 0.5. This experiment was conducted 30 days after callus induction with the basal CIM.

2.5. Combination of the effective CIM and SIM with WPM or LP media

Once effective combinations CIM and SIM were identified, we moved to evaluate PGRs combinations of the two steps. Leaf explants were first cultivated on CIM with three different PGRs combinations: 0.5 µM TDZ + 0.5 µM NAA, 2.5 µM BAP + 0.5 µM NAA and 0.25 µM TDZ + 2.5 µM BAP, and then transferred to SIM with 5.0 µM BAP + 1.0 µM NAA. This experiment was conducted using WPM or LP (Quoirin & Lepoivre 1977) to compare between the culture medium.

2.6. In vitro rooting and acclimatization

Shoot explants (1 cm of length) were collected and inoculated on test tubes with 10 mL of 1/4 MS (full strength of vitamins) containing 15 gL^{-1} sucrose, 1 mg L^{-1} riboflavin and 7.0 gL^{-1} agar. In this experiment, five concentrations of IBA (Indole-butyric acid) (1, 2, 3, 4 and 5 mg L^{-1} corresponding to 4.9, 9.8, 14.7, 19.6 and 24.5 µM) were compared. The explants were incubated in the dark for 10 days and then transferred to light for another 45 days. The experiment consisted of 30 replications per treatment. After two months, the percentage of root formation, number of secondary roots, length of the main root and stem elongation were evaluated. To acclimatize the rooted explants, the test tubes were partially opened and incubated in the growth room for two days. Following that, the roots were rinsed to remove agar and transferred to plastic pots containing autoclaved soil-vermiculate mixture (1:1) and kept in a greenhouse. The percentage survival was recorded after 45 days.

2.7. Genetic transformation via Agrobacterium tumefaciens

Leaves of *in vitro* grown plants were used. The explants were placed on pre culture medium (basal CIM with 0.5 µM of TDZ + 0.5 µM NAA) for 1 day in the dark. Following that, explants were co-cultivated on ½ MS liquid medium containing a bacterial suspension of *A. tumefaciens* strain EHA105 containing pCAMBIA 2301

vector (Fig 1), containing *nptII* selection gene and *uidA* gene, both under control of the CaMV35S promoter. The bacteria was previously grown overnight in a LB (SAMBROOK *et al.*, 1989) (10.0 gL^{-1} Tryptone, 10.0 gL^{-1} NaCl and 5.0 gL^{-1} yeast extract, pH 7.0) medium supplemented with 50 mgL^{-1} of kanamycin at 28°C and120 rpm until it reached $OD600_{nm}$ 0,5-1.

After co-culture, the explants were transferred to CIM medium with containing 50 mgL^{-1} kanamycin for three weeks and were then transferred to SIM medium (with 5 µM BAP + 1 µM NAA) supplemented with 100 mgL^{-1} kanamycin. The shoots regenerated on kanamycin were transferred to multiplication medium (see section 2.1.) supplemented with 150 mgL^{-1} kanamycin. We used 500 explants for transformation and 50 explants as control without passing through genetic transformation protocol.

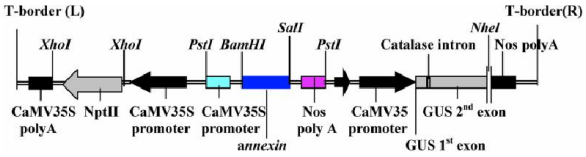

Figure 1. Pcambia 2301 vector scheme. Source: YADAV *et al.*, (2012).

2.8. Gene insertion confirmation and gene expression evaluation

For PCR analysis to confirm gene insertion, DNA samples from the putative transgenic shoots as well a control explant were extracted by CTAB 2% (Catonic Hexadecyl Trimethyl Ammonium Bromide) protocol (FERREIRA and GRATTAPAGLIA, 1998) and amplified using the forward and reverse primes of *uidA* (F: 5´-CAGCGCGAAGTCTTTATACCG-3´; R: 5´-ATGCGTCACCACGGTGATATCG-3´) and *nptII* genes (F: 5´-TCGGCTATGACTGGGCACAACAGA-3´; R: 5´-AAGAAGGCGATAGAAGGCGATGCG-3´) (the amplicon size were 368 bp and 941 bp, respectively). The PCR mixture (25 µl) contained 1.0 U *Taq* DNA polymerase, 2.0 mM $MgCl_2$, 1x PCR buffer, 2.0mM of each dNTP, 0.4 mM of each forward and reverse primer (Invitrogen™, Br) and approximately 50 ng template DNA. For positive control 4.0 ng of pCAMBIA 2301 plasmid DNA was used. The PCR was performed using a Veriti 96 Well Thermal cycler from Applied Biosystem, and consisted of: 1 cycle at 94°C for 5 min, 30 cycles at 94°C for 1 min, 65°C for 1 min and 72°C for 2 min, 1 cycle at 72 °C for 7 min for *nptII* gene and 1 cycle at 94°C for 4 min, 35 cycles

at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, 1 cycle at 72°C for 7 min for *uidA* gene. The amplification products were separated by 1% agarose gel electrophoresis with ethidium bromide solution (0.5 mg/ml) (Invitrogen™, Br) in 1X buffer TBE (0.04 M TRIS-acetate, 1 mM EDTA, pH= 8) at 80 V for 1 hour. The gel was photographed using a Cannon PowerShot S2 IS camera under Syngene Bio Imaging program.

To confirm the gene expression, histochemical analyses were performed as described by Jefferson (1987) of the putative transgenic shoots as well as a control explant.

2.9. Somaclonal variation

Leaves were collected from the plants under micropropagation for 1.5 years and shoots derived from indirect organogenesis cultured on three different PGRs combinations and concentrations, as described on the first experiment (treatments T7, T15 and T17, Table 1). DNA was extracted according to Ferreira & Grattapaglia (1998) using CTAB 2%. RAPD profiles were generated using 11 arbitrary primers (Biolegio Co., Ho) (sequences are shown in Table 4). The amplification was performed in a DNA Thermal Cycler (Apllied Biosystem™) according to the following program: 1 cycle of 4 min at 94°C, 40 cycles of 1 min at 94°C, 1.30 min at 36°C, and 1.30 min at 72°C, and finally 1 cycle of 7 min at 72°C. The amplification was carried out in a 96 well Microplate in a volume of 25µl containing approximately 25 ng of DNA template, 1x of PCR buffer, 100 µM of each dNTP, 2 µM of Mg^{2+} , 36.5 µM of primer and 1 unit of *Taq* DNA Polymerase, provided by Invitrogen™. The analyses were loaded in a QIAxcel Advanced system and read by QIAxcel ScreenGel program (Quiagen®). All the amplifications were done in triplicate, and only bands replicated in at least on two samples, with a medium to strong intensity, a difference of ± 20 base pairs per sample and above 1 ng/µg concentration, were scored. The specific amplification was scored as present (1) or absent (0).

2.10. Evaluations and Statistical analysis

The experimental designs were completely randomized, with two replicates with 40 explants per each petri dish and repeated twice. After 30 and 90 days oxidation, presence of anthocyanin, callus formation and shoot formation in explants with callus and number of shoots per explants (the last one only after 90 days) were

evaluate. The data analysis of the experiments was performed by analysis of Deviance for the model that considered the effect of treatments. The binary data were analyzed by generalized linear models with binomial distribution probability and for the quantitative data Poisson distribution was used. Comparisons between treatments were made by Tukey test ($p<0.05$). The data were analyzed by the R software environment statistic program (R DEVELOPMENT CORE TEAM, 2011).

3. RESULTS

3.1. Establishment of a two-step organogenesis protocol

Step 1: Callus induction medium

We evaluated the efficiency of a two-step organogenesis protocol for regeneration of clone BRS07-01 from leaves of *in vitro* grown plants. In the first step, we evaluated effects of PGRs combinations and concentrations on callus and shoot formation. Higher callus formation was observed on explants cultured on TDZ combined either with NAA or 2,4-D relative to BAP after 30 and 90 days, reaching up to 81.2% of the total (Table 1). However, when TDZ and NAA are analyzed alone, we observed that at lower concentrations of NAA, callus induction was reduced, regardless the concentration of TDZ tested.

Regarding BAP, only the lower concentration (2.5 µM) was effective for callus formation and it was as effective as TDZ. The same pattern of callus formation was observed among the treatments after 30 and 90 days, showing the influence of PGRs of the first step even after shoot formation (Table 1).

For shoot formation the same results were observed as for callus formation, which TDZ proved to be more effective than BAP at high concentrations. Higher concentrations of BAP seem to have a deleterious effect on shoot induction in clone BRS07-01 (Table 1). The best shoot formations were observed on the treatments with TDZ and both auxins, reaching up to 47.5%, when BAP was used in lower concentration (2.5 µM) with NAA, also with 47.5% and when both citokynins TDZ and BAP were combined, resulting in 48.1% of shoot induction. Although none of this treatments have differed statistically.

TABLE 1 - Comparison of different concentrations and combinations of PGRs on indirect organogenesis of *E. urophylla* clone BRS07-01. Data analysis after 30 days for percentage of callus proliferation, shoot induction, presence of anthocyanin and after 90 days for percentage of callus proliferation, shoot induction, number of shoots per explant and presence of anthocyanin. After 30 days the explants where transferred to SIM for further culture.

Treatments (in µM)		After 30 days		After 90 days			
		Callus formation	Anthocyanin	Callus formation	Shoots induction	No. Shoots per explant	Anthocyanin
T1	0.25 TDZ + 0.1 NAA	23.8±9.4^{cde}	25.0±9.5^{bcd}	38.8±10.7^{c}	28.8±10.0 ^{bc}	2.976±0.53^{abc}	30.0±10.1^{ab}
T2	0.5 TDZ + 0.1 NAA	17.5±8.4 ^{de}	21.2±9.0 ^{bcd}	46.0±14.0 ^{c}	32.0±13.1 ^{bc}	3.325±1.103^{abc}	34.0±13.3^{a}
T3	0.75 TDZ + 0.1 NAA	22.5±9.2 ^{de}	16.2±8.1^{cde}	51.2±11.0 ^{bc}	27.5±9.8 ^{bc}	3.605±0.307 ^{abc}	30.0±10.1^{ab}
T4	1.0 TDZ + 0.1 NAA	31.2±10.2^{bcde}	32.5±10.3^{abc}	51.2±11.0 ^{bc}	36.2±10.6 ^{ab}	4.259±1.789 ^{ab}	26.2±9.7 ^{abc}
T5	2.0 TDZ + 0.1 NAA	37.5±10.7^{abcd}	3.8±4.2^{ef}	73.8±9.7 ^{ab}	32.5±10.3 ^{bc}	2.832±1.116 ^{abc}	18.8±8.6 ^{abc}
T6	0.5 TDZ + 0.2 NAA	56.2±10.9^{a}	46.2±11.0^{a}	73.3±7.9 ^{ab}	36.7±8.7 ^{ab}	4.036±1.637 ^{ab}	34.2±8.5 ^{a}
T7	0.5 TDZ + 0.5 NAA	56.0±11.3^{ab}	40.0±11.2^{ab}	81.2±6.1 ^{ab}	47.5±7.8 ^{a}	5.075±1.087 ^{a}	9.4±4.5 ^{bc}
T8	0.5 TDZ + 0.1 2,4D	53.8±11.0^{abc}	18.8±8.6^{bcde}	71.7±8.1 ^{ab}	40.8±8.8 ^{ab}	3.767±0.548 ^{abc}	28.3±8.1 ^{abc}
T9	0.5 TDZ + 0.2 2,4-D	51.2±11.0^{abc}	17.5±8.4 ^{bcde}	79.4±6.3 ^{ab}	35.6±7.4 ^{ab}	3.311±0.523 ^{abc}	33.8±7.4 ^{a}
T10	5.0 BAP + 0.1 NAA	16.2±8.1 ^{de}	0±0^{f}	57.7±9.5 ^{b}	30.8±8.9 ^{bc}	2.332±0.34 ^{bc}	9.6±5.7 ^{bc}
T11	5 BAP + 0.5 NAA	16.9±8.4 ^{de}	6.5±5.5^{de}	50.6±7.8 ^{bc}	23.8±6.6 ^{bc}	2.611±0.430 ^{bc}	20.6±6.3 ^{abc}
T12	5 BAP + 1.0 NAA	14.1±7.8 ^{e}	0±0^{f}	55.6±8.1 ^{bc}	25.0±7.1 ^{bc}	2.525±0.378 ^{bc}	22.9±6.9 ^{abc}
T13	5 BAP + 2.5 NAA	16.2±8.1 ^{de}	15.0±7.9^{cde}	66.2±7.4 ^{b}	25.6±6.8 ^{bc}	3.289±0.876 ^{abc}	16.2±5.7 ^{abc}
T14	7.5 BAP + 0.5 NAA	12.5±7.3^{e}	0±0^{f}	57.5±8.9 ^{b}	15.0±6.4 ^{c}	1.550±1.099 ^{c}	4.2±3.6 ^{c}
T15	2.5 BAP + 0.5 NAA	38.8±10.7^{abcd}	28.8±10.0^{abc}	71.2±7.0 ^{ab}	47.5±7.8 ^{a}	3.105±0.568 ^{abc}	17.5±5.9 ^{abc}
T16	7.5 BAP + 0.1 NAA	17.5±8.4^{de}	12.5±7.3^{cde}	51.1±8.4 ^{bc}	35.0±8.0 ^{bc}	2.202±0.347 ^{bc}	6.6±4.2 ^{c}
T17	0.25 TDZ + 2.5 BAP	31.2±10.2^{bcde}	12.5±7.3^{abc}	73.8±6.8 ^{ab}	48.1±7.8 ^{a}	3.607±0.851 ^{abc}	25.6±6.8 ^{abc}

Values followed by the same letter between the lines does not differ by Tukey test ($p<0.05$).

We observed the presence of anthocyanin pigmentation on callus in almost all treatments as demonstrated on Figure 2A. The oxidation ranged from 86% to 100% after 30 days on culture and was observed in all treatments.

Step 2: Shoot induction medium (SIM)

Calluses previously cultivated on the control CIM medium (supplemented with 0.5 µM TDZ + 0.1 µM NAA) were cultured on SIM medium with different concentrations of BAP and NAA in order to improve the shoot induction.

The higher shoot formation was observed on 5.0 µM of BAP and 1.0 µM NAA (63.8%) with an average of 5.667±0.835 shoots per explant (Figure 2B), although not differing statistically from the other treatments, except from the treatment with the lower concentration of BAP (Table 2).

The lower shoot formation frequency was observed on 2.5 µM BAP combined with 0.5 µM NAA (25.49%), suggesting that higher concentrations of BAP are necessary to increase the regeneration frequency (Table 2).

Oxidation was observed in all treatments, and it was higher in the worst treatment, when all explants oxidized. These results suggest that oxidation can be related to a lower rate of response, but we observed that even oxidized callus were able to regenerate.

Table 2 - Effect of BAP and NAA in shoot induction medium (SIM) of E. urophylla clone BRS07-01, after 90 days of culturing. The explants were first cultured on CIM to promote callus formation.

Treatment	BAP (µM)	NAA (µM)	Shoots induction	No. Shoot per explant	Oxidation
T1	5.0	0.1	46.2±11.0^{ab}	5.378±1.197^{a}	55.0±11.0^{d}
T2	5.0	0.5	43.8±10.9 ^{ab}	4.111±0.916^{ab}	82.5±8.4^{c}
T3	5.0	1.0	63.8±10.6^{a}	5.667±0.835^{a}	80.0±8.8^{c}
T4	2.5	0.5	25.5±12.1^{b}	3.236±1.968^{b}	100.0±0^{a}
T5	7.5	0.5	43.4±11.2 ^{ab}	4.958±1.022^{ab}	97.4±3.6^{b}

Values followed by the same letter between the lines does not differ by Tukey test ($p<0.05$).

3.2. Combination of the best CIM and SIM with WPM or LP

We previously tested three different culture media and LP demonstrated higher shoot induction (data not shown). For this reason we decided to evaluate the more effective PGRs combinations on this medium and compared it to WPM.

However, more callus and shoot induction was observed on WPM medium (Table 3). Higher shoot induction was observed on 0.5 µM TDZ + 0.5 µM NAA (85.6%), with 8.64±0.835 shoots per explant, differing from all treatment with LP and from WPM supplemented with BAP and NAA combined (for shoot induction). The lower callus and shoot induction and higher oxidation rate were observed on LP supplemented with 2.5 µM BAP + 0.5 µM NAA.

The combination of TDZ and BAP on CIM media, respectively, was more effective than BAP combined with the auxin on the organogenesis of this clone. The number of shoots per explant showed a high variation among the treatments, ranging from 0.7 to 8.64 in the best treatment.

Table 3- Evaluation of *in vitro* regeneration of Clone BRS07-01 of *E. urophylla*, cultured on the best CIM and SIM PGRs concentrations and combinations on WPM or LP. Callus formation percentage evaluation after 30 and callus, shoot induction, shoot per explant, oxidation and anthocyanin percentage after 90 days.

Media/ Treatment	After 30 days	After 90 days			
	Callus	Callus	Shoots	No. Shoots per explant	Oxidation
WPM T1	76.2±4.8^{a}	94.3±6.6^{a}	85.6±5.5^{a}	8.64±0.835^{a}	25.0±7.0^{e}
WPM T2	55.6±13.3^{b}	75.6±9.7^{ab}	33.7±5.2^{b}	3.15±1.41^{c}	76.2±12.3^{b}
WPM T3	65.6±7.5^{ab}	72.5±10.2^{b}	66.2±7.2^{a}	5.26±0.331^{bc}	48.1±8.5^{d}
LP T1	76.8±10.0^{a}	82.5±6.5^{ab}	35.0±14.2^{b}	6.07±1.137^{b}	68.1±10.7^{bc}
LP T2	33.1±2.4^{c}	49.3±7.5^{c}	31.3±6.2^{c}	0.7±1.4^{d}	99.4±1.25^{a}
LP T3	57.5±11.4^{ab}	69.2±10.3^{b}	39.1±13.6^{b}	6.31±0.182^{b}	57.5±3.5^{cd}

The explants were cultured first on CIM supplemented with: T1 – 0.5 µM TDZ + 0.5 µM NAA; T2 – 2.5 µM BAP + 0.5 µM NAA; T3 – 0.25 µM TDZ + 2.5 µM BAP, and the transferred to SIM with 5.0 µM BAP + 1.0 µM NAA. Values followed by the same letter between the lines does not differ by Tukey test ($p<0.05$).

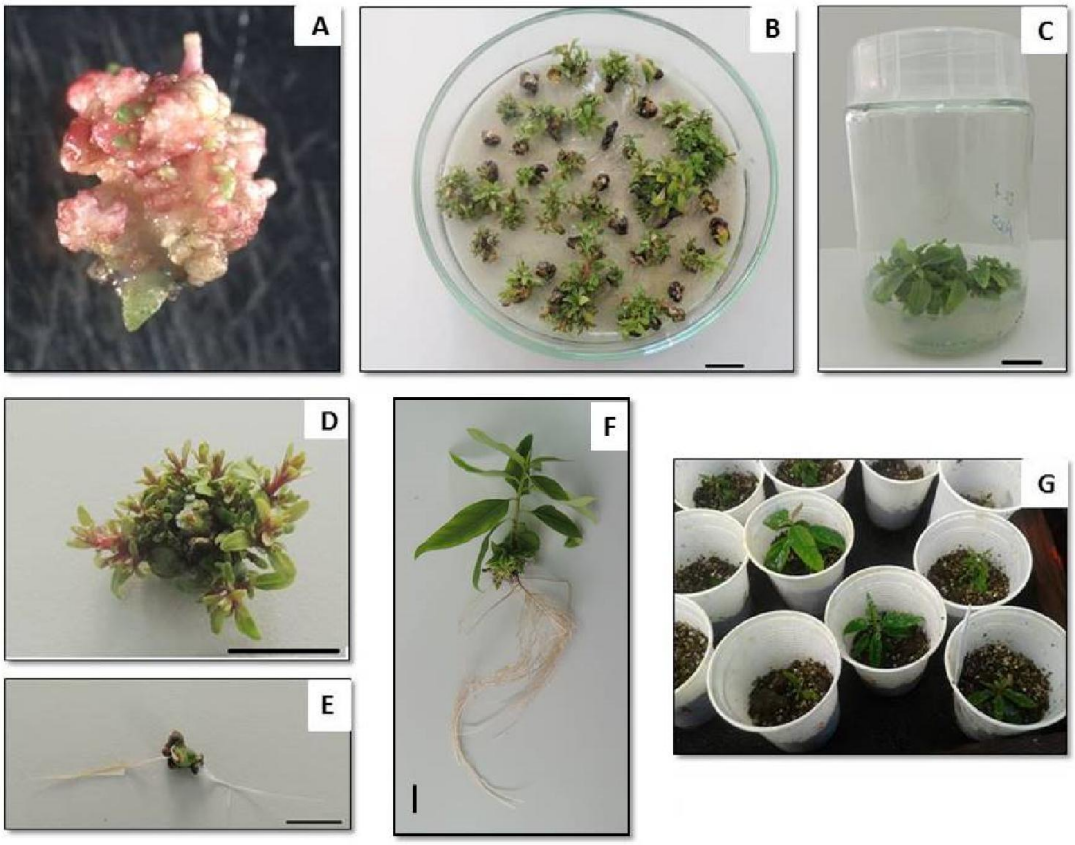

Figure 2. Indirect organogenesis of *E. urophylla* clone BRS07-01. (**A)** Callus formation after 30 days of culture on WPM + 0.5 µM TDZ + 0.1 µM NAA; (**B)** Shoot induction of explants after 90 days, prior cultivated on WPM + 0.5 µM TDZ + 0.1 µM NAA for callus induction and then transferred to WPM medium with 5.0 µM BAP + 0.5 µM NAA; **(C)** Microshoots micropropageted *in vitro* Explants; **(D)** Explants with adventitious shoots cultivated on WPM + 5.0 µM BAP + 1.0 µM NAA; **(E)** Explants with adventitious root cultivated on WPM + 2.5 µM BAP + 0.5 µM NAA; **(F)** Plantlets showing adventitious roots cultivated on 4.9 µM IBA; G) Rooted plantlets after 3 weeks acclimatized on greenhouse. Bar = 1cm

3.3. Genetic transformation

After selection on kanamycin, the shoots were transferred to multiplication medium. Two months later, DNA from leaves was extracted and the insertion was confirmed by PCR (Figure 3A). The wild type plantlet did not show the bands. Expression of the *gus* gene was confirmed by blue staining of the plantlets (Figure 3B).

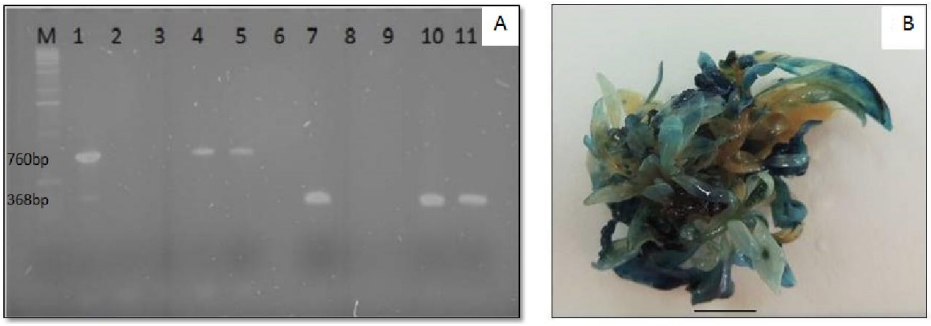

Figure 3. **(A)** *nptII* and *uidA* PCR amplification from two transformants events of Clone Brs07-01. Legend: M- 1kb marker, 1- Positive control of *nptII* vector, 2- Blank, 3- Negative control, 4 and 5 - positive events for *nptII* gene, 7 - Positive control of *uidA* vector, 8- Blank, 9- Negative control, 10 and 11 - positive events for *gus*. **(B)** Transient *gus* staining on shoot tip derived from *Agrobacterium*-mediated genetic transformation of clone Brs07-01. Bar = 0.5cm.

3.4. In vitro rooting and acclimatization

We evaluated five different concentrations of IBA on the rooting of clone BRS07-01. Root formation was higher when 14.7 µM of IBA was used, reaching up to 84.0% and differing statistically from all treatments (Table 4). The averages of the main root length and stem elongation were also higher with 14.7 µM of IBA, with 4.7 cm and 1.60 cm, respectively. Although not differing statistically with 19.8 µM for root length (2.9 cm) and stem elongation (0.89 cm) and 24.5 µM for stem elongation (0.98 cm). However, the differences were statistically insignificant. Approximately 80% of the plantlets were successfully transferred to the greenhouse (Figure 2G).

Table 4- Evaluation of *in vitro* rooting of Clone BRS07-01 of *E. urophylla*, cultured on ¼ MS medium supplemented with different concentrations of IBA (4.9, 9.8, 14.7, 19.6 and 24.5 µM). Roots percentage, primary root length and explant elongation were evaluated after 45 days.

IBA (µM)	Root formation (%)	Main root Length (cm)	Stem Elongation (cm)	Number of seconds root
4.9	13.3±8.3^{c}	0.6±0.352^{b}	0.23±0.112^{b}	0.9±0.508^{b}
9.8	29.6±8.7^{bc}	1.4±0.348^{b}	0.54±0.231^{b}	1.5±1.135^{b}
14.7	84.0±9.1^{a}	4.7±0.355^{a}	1.60±0.230^{a}	7.9±1.40^{a}
19.6	37.0±8.7^{bc}	1.8±0.359^{b}	0.89±0.294^{ab}	3.0±0.90^{b}
24.5	50.0±8.6^{b}	2.9±0.197^{ab}	0.98±0.222^{ab}	3.5±1.017^{b}

Values followed by the same letter between the lines does not differ by Tukey test ($p<0.05$).

3.5. Somaclonal variation

Our results showed that our clone preserved its stability during the in vitro culture. We tested 11 RAPD primers for somaclonal variation in the samples of plants under micropropagation for 2 years, shoots derived from indirect organogenesis on different PGRs and from *in vitro* rooted plantlets. We evaluated 56 markers. The size of the markers ranged from 120-1580 bp.

From the 11 universal primers tested, we could not observe any polymorphic band among the different *in vitro* derived shoots and the plants under micropropagation which served as source for the explants used at the experiments. In addition, the lack of somaclonal variation also indicated that the clones preserved their integrity.

Table 5- Sequence of the primers used in RAPD of the *E. urophylla* explants and numbers of bands amplified and their size range (±50pb).

Primers	Sequences (5' →3')	No. of bands	Range of the bands (bp)
OPA-13	AAGCCTCGTC	3	185-1400
OPA-17	GACCGCTTGTC	3	386-1000
OPA-18	AGGTGACCGT	5	121-884
OPC-01	TTCGAGCCAG	2	1175-1790
OPC-02	GTGAGGCGTC	4	277-941
OPC-04	CCGCATCTAC	6	341-1580
OPC-06	GAACGGACTC	4	251-1010
OPC-07	GTCCCGACGA	14	170-946
OPC-19	GTTGCCAGCC	6	221-960
OPP-03	CTGATACGCC	2	760-960
OPP-04	GTGTCTCAGG	6	232-1375
Total Bands		56	121-1580

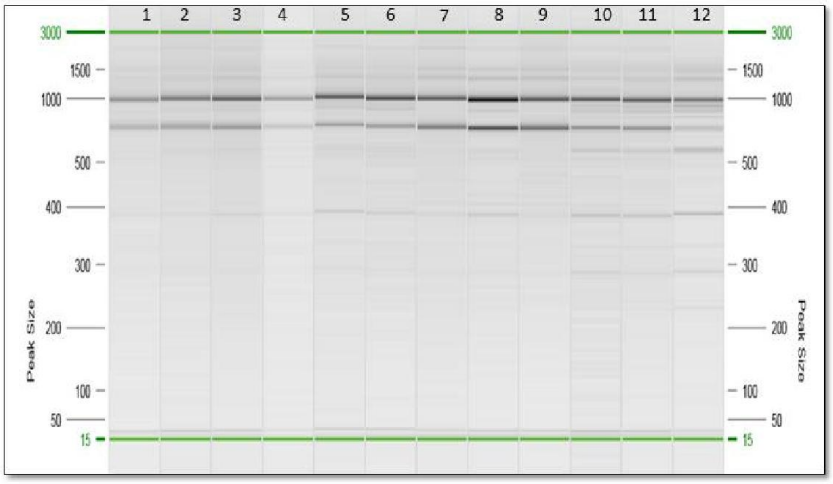

Figure 4. Polymorphims bands of RAPD resulted from OPA-17 primer of shoots derived from indirect organogenesis: 1-3: 0.5 µM TDZ + 0.5 µM NAA; 4-6: 2.5 µM BAP + 0.5 µM NAA; 7-9: 0.25 µM TDZ + 0.25 µM BAP, and 10-12: plants under micropropagation, generated by QIAxcel ScreenGel 1.2.0 (Quiagen®).

4. DISCUSSION

4.1. Two-step organogenesis protocol

The use of two step regeneration protocols for *Eucalyptus* was introduced by Bandypadhyay *et al.* (1999) for *E. nitens* and *E. globulus* and have being used since for *Eucalyptus* species in most of the protocols (CHITRA and PADMAJA, 2005; OLIVEIRA *et al.*, 2015). Often, the steps differ in their PGRs types and concentrations, and normally the cytokinin concentration is increased in the second step (BANDYPADHYAY *et al*., 1999).

Once it is well known that TDZ should be used at lower concentrations in order to compare the regeneration results with other citokynins (NORTH *et al.*, 2012), we used it diluted 10 fold when compared to BAP, and TDZ was in general more efficient on callus induction than BAP (Table 1). This is in line with observations of higher callus induction with TDZ when compared to BAP for *E. grandis* × *E. urophylla* (ALVES *et al.*, 2004; BARRUETO CID *et al.*, 1999), *E. grandis* and *E. urograndis (*GONZÁLEZ, 2002). Similar results were also observed for another elite clone of *E. urograndis* (AEC 224 clone), for which 0.25 µM TDZ and 0.1 µM NAA induced higher callus formation compared with 5.0 µM BAP and 0.5 µM NAA (OLIVEIRA *et al.*, 2015).

TDZ was observed to stimulate axillary shoot proliferation in higher rates than most cytokinins even in lower concentrations (HUETTEMAN and PREECE, 1993). It is believed to mimic cytokinin-like activity by promoting endogenous cytokinin synthesis and/or accumulation in various morphogenetic responses, including shoot stimulation in a range of recalcitrant species (RUŽIĆ and VUJOVIĆ, 2008; AGGARWAL *et al.*, 2012b).

Although 5.0 or 7.5 μM of BAP resulted in lower callus formation, 2.5 μM of BAP did not differ statistically from the best media with TDZ (Table 1). Glocke *et al.* (2006) and Dibax *et al.* (2005) also reported efficient callus induction of *E. camaldulensis* (over 90%) using BAP alone or in combination with NAA. BAP was also reported by Hajari *et al.* (2006) to induce 100% callus formation on *E. grandis* and *E. grandis* × *E. urophylla* clones when combined with IAA. However, these studies did not test TDZ.

Interestingly, a combination of BAP and TDZ without auxins was equally potent in inducing callus and shoot formation. This result suggests rather that auxins are needed in lower concentrations or that TDZ can be acting also as an auxin in the regeneration of our clone. Murthy & Murch (1998) and Suttle (1988) suggested that TDZ mimics auxin-like metabolic responses, not acting directly as an auxin, but by inducing and increasing the auxin response. Azmi *et al.* (1997) also showed that combinations of two cytokinins can induce callus formation and bud initiation. They reported about 70% regeneration from hypocotyls and 55% from cotyledons of *E. globulus* Labill explants using a combination of 6-Bezyladenine (BA) and TDZ.

The results of the first experiment showed that the combinations and concentrations of the PGRs had influenced the shoot induction response even after that all the explants were transferred to SIM medium. Our shoot induction rates (reaching up to 48.1%) were higher than those observed by Oliveira-Cauduro *et al.* (2014) for an *E. benthamii* x *E. dunnii* clone (8.3%) ; Hervé *et al.* (2001) for an *E. gunni* clone (8.8%), Dibax *et al.* (2010a) for *E. saligna* (40%) and Aggarwal *et al.* (2010) on *E. tereticornis* (40.5%). However, these species are related to be even more recalcitrant to in vitro culture than *E. urophylla.*

The NAA concentration was primordial for callus and shoot induction when combined to TDZ. On the last callus evaluation on table 1 we can observe that the lower concentration of NAA tested (0.1 μM) was more effective as when

used on the range of 0.2-0.5 μM, only when combined with the higher concentration of TDZ (2.0 μM).

Auxins acts crucially in the dedifferentiation and differentiation of cultured tissues (MURTHY and MURCH, 1998). The synthetic auxins NAA and 2,4-D are considered to be stable and they differ in their physiological activity and in the extent to which they translocate through tissue and are metabolized (SAAD and ELSHAHED, 2012).

Alves *et al.* (2004) also reported higher callus formation on 0.54 μM NAA, although combined with a higher concentration of TDZ (2.27 μM) for *Eucalyptus grandis x E. urophylla* clones. However, different from our approach, most of the protocols of *Eucalyptus* indirect organogenesis use higher concentrations of NAA (RIBEIRO, 2012; DIBAX *et al.*, 2010a; MULLINS *et al.*, 1997, MURALIDHARAN & MASCARENHAS, 1987). Increasing NAA concentration also enhanced callus formation on *E. grandis* indirect organogenesis (RIBEIRO, 2012). Which concentrations from 2.69-16.11μM of NAA alone or combined with 2,4-D and Zeatine, have resulted in nearly 100% of callus formation.

Dibax *et al.* (2005) reported efficiency of callus induction using BAP alone or in combination with NAA for *E. camaldulensis,* and they obtained higher callus induction using 2.7 or 5.4 μM NAA and 4.44 μM BAP (over 90%). Some studies also report higher callus formation on media with higher concentration of auxin than cytokinin, normally BAP (HO *et al.*, 1998; DIALLO and DUHOUX, 1984; MULLINS *et al.*, 1997). Although we did not test this ratio inversion, our results show that the effect of NAA in concentrations higher than 0.2 μM does positively influence the callus induction for our clone.

We observed red pigmentation, probably anthocyanin, in some explants (Figure 2A) in line with previous reports by Bandypadhyay *et al.* (1999) on *E. nitens* callus. The synthesis and accumulation of these pigments in plants are related to photoinhibition (NAVARRO *et al.*, 2011) to confer photoprotection (CLOSE *et al.*, 2003). Anthocyanin accumulation is broadly related to abiotic stress responses (NAVARRO *et al.*, 2011). We also observed higher organogenesis rates in tissue sections that showed higher anthocyanin accumulation (visually assayed from the coloration density) as described by Dibax *et al.* (2005) for *E. camaldulensis* and Hervé *et al.* (2001) for *E. gunni*.

These results suggest that a stress source could positively influence the differentiation of the callus cell into a new meristematic tissue.

In the second step (SIM), we avoided TDZ since when used for long periods, it has a detrimental effect on rooting (MUNDHARA and RASHID, 2006; PREECE and IMEL, 1993; TSURO *et al.*, 1999). In order to enhance shoot formation, we tested concentrations and combinations of BAP and NAA that have previously being described as efficient for other *Eucalyptus species* (AGGARWAL *et al.*, 2010; AHAD *et al.*, 2014). BAP is the most frequently cytokinin used for shoot induction. This cytokinin increases the conversion efficiency of undifferentiated cells into regenerated shoots and is important frequently used to avoid TDZ continuously during the culture (MYCOCK and WATT, 2012).

The lower shoot frequency was observed on one of the best CIM media: 2.5 µM BAP combined with 0.5 µM NAA (25.49%), suggesting that after 30 days, higher concentrations of BAP are necessary for shoot induction. This was also observed by Aggarwal *et al.* (2010) for *E. tereticornis.* And also the shoot induction increased when the NAA concentration was increased to 1.0 µM (63.8%).

Similar to our experiments, Alves *et al.* (2004) also evaluated TDZ and BAP in combination with NAA for callus induction followed by BAP and NAA for shoot induction for *Eucalyptus* clones. In their work, one of the clones showed high shoot response when cultured on 4.44 µM BAP without auxin and another one on 2.22 µM BAP and 1.07 µM NAA, but the shoot regeneration efficiency was very lower than ours (8% regeneration). Different SIM media were also tested by Bandypadhyay *et al.* (1999), where the combination of 2.69 µM NAA and 4.44 µM BAP allowed high frequency of plant regeneration from cotyledons and hypocotyls of both *E. nitens* and *E. globulus*.

The effect of combining the most efficient CIM and SIM can be observed on Table 3. The combination of 0.5 µM of TDZ with 0.5 µM of NAA on CIM; and 5.0 µM BAP and 1.0 µM NAA on SIM medium allowed up to 85.6% of shoot regeneration. To our knowledge, no one has ever reported regeneration above of 60% for a *Eucalyptus* species clone by indirect organogenesis. Regeneration rates of 53% were observed for *E. grandis* × *E. urophylla* (BARRUETO CID *et al.*, 1999), 47-57.5% for cotyledonary leaves of *E.*

camaldulensis (DIBAX *et al.*, 2005; DIBAX *et al.*, 2010b), 40% for cotyledonary explants of *E. saligna* (DIBAX *et al.*, 2010a), 50% for cotyledonary of *E. globulus* sp. (SERRANO *et al.*, 1996) and 52% for *E. grandis* clone (MA *et al.*, 2011). However, it is noteworthy to point that most of these works used seeds as sources of explants instead of a clone.

Although oxidation was observed in all treatments, it does not seem to influence organogenesis. Generally, the oxidation rates were over 90% in all treatment (data not shown) and can be related to the production and accumulation of the phenolic exudates produced by the wounded plant tissue or to the exposure to light (NDAKIDEMI *et al.*, 2014). Bravo (2005) observed absence of oxidation during cultivation in the dark on *E. grandis* and Barrueto Cid *et al.* (1999) observed oxidation on the explants of *E. grandis* × *E. urophylla* cultured for more than 8 weeks and correlated it to the activation of specific promoters involved in the biosynthesis of phenolic compounds by light.

4.2. Rooting and acclimatization

We tested the effect of five IBA concentrations on rooting of clone BRS07-01. Root formation was observed in up to 84.0% of explants cultivated on medium containing 14.7 µM of IBA. Tournier *et al.* (2003) reported 100% of success of *E. urophylla* rooting, reaching of survival and which observed 95% of survivor rate using a sand and fertilizer mixture under greenhouse conditions. However, our rooting rates were higher than those observed by Rahim *et al.* (2003) (20%) for *E. camaldulensis* and by Oliveira for *et al.* (2015) for *E. urophylla* (35%) using 2.45 µM IBA.

After rooting, up to 80% of the plants were successfully acclimatized. This result is in accordance with previous studies on various *Eucalyptus* species and clones (MYCOCK and WATT, 2012; OLIVEIRA *et al.*, 2015).

4.3. Genetic transformation of clone BRS07-01

It is widely acknowledged that *Eucalyptus* species are very recalcitrant to genetic transformation. In order to test the transformation potential of clone BRS07-01, we tested an optimized genetic transformation protocol via *Agrobacterium tumefaciens* for this clone (BETTENCOURT *et al.*, under preparation).

Using a pCAMBIA2301 vector, we were able to regenerate plantlets on medium supplemented with kanamycin with an efficiency of 20.83%. The integration of the *gus* and *nptII* genes was confirmed by PCR and the *gus* expression verified by histochemical analysis (Figure 3). This result reinforces our hypothesis that the BRS07-01 clone can be used as a model plant for transformation studies in *Eucalyptus*.

4.4. Somaclonal variation

Media components (PGRs, antibiotics, etc) (MUNIR *et al.*, 2011; SUN *et al.*, 2013) as well as the number and duration of sub-cultures (LEVA *et al.*, 2012) have been shown to induce genetic changes *in vitro* culture. Therefore, it is important to verify that the genetic integrity of clones has been conserved after the organogenesis steps.

We assessed explants originated from indirect organogenesis and those cultured on growth room under micropropagation for somaclonal variation using RAPDs. Even though the technique has some limitations, it is relatively cheap and easier to execute than other molecular markers, like SSR Microsatellite (Simple sequence repeat) (POKE *et al.*, 2005; LEVA *et al.*, 2012; MUNIR *et al.*, 2011). In all the in vitro stages, the shoots and plants under micropropagation showed the same RAPD pattern for all the 56 bands assessed. This result indicates the genome stability and uniformity of our clone. This result is also consistent with earlier studies by Aggarwal *et al.* (2010) and Aggarwal *et al.* (2012a), where no somaclonal variation was detected for *E. tereticornis* explants using RAPD markers.

5. CONCLUSION

We obtained a regeneration protocol with an efficiency of up to 85.6% shoot formation for the clone BRS07-01 of *Eucalyptus urophylla*. The clone was sucessfully transformed via *Agrobacterium tumefaciens*. Although the rooting protocol still must to be improved, we suggest that this clone could be used in functional genomic studies, for validation of gene function. According to the analysis of 56 RAPD markers, we did not observe any somaclonal variation for this clone during indirect organogenesis culture.

Acknowledgments

The authors wish to thank Dr. Estefano Paludzyszyn Filho and Paulo Eduardo Telles dos Santos from Embrapa Forestry, for providing the clone BRS07-01 and the technical information about it. We would also like to acknowledge CAPES for the Master scholarship (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and EMBRAPA Forestry (Empresa Brasileira de Pesquisa Agropecuária) for the financial support.

6. REFERENCES

AGGARWAL, D.; KUMAR, A.; REDDY, M. S. Shoot organogenesis in elite clones of Eucalyptus tereticornis. **Plant Cell, Tissue and Organ Culture**, v. 102, n. 1, p. 45–52, 2010.

AGGARWAL, D.; KUMAR, A.; SHARMA, J.; REDDY, M. S. Factors affecting micropropagation and acclimatization of an elite clone of Eucalyptus tereticornis Sm. **In Vitro Cellular and Developmental Biology - Plant**, v. 48, n. 5, p. 521–529, 2012a.

AGGARWAL, G.; SHARMA, C.; SRIVASTAVA, D. K. Thidiazuron: A potent cytokinin for efficient plant regeneration in Himalayan poplar (Populus ciliata Wall.) using leaf explants. **Ann. For. Res.**, v. 55, n. 2, p. 179–188, 2012b.

ALCANTARA, G. B. DE; BESPALHOK FILHO, J. C.; QUOIRIN, M. Organogenesis and transient genetic transformation of the hybrid Eucalyptus grandis × Eucalyptus urophylla Organogênese e transformação genética transiente do híbrido Eucalyptus grandis × Eucalyptus urophylla. **Scientia Agricola**, v. 68, n. 2, p. 246–251, 2011.

ALVES, E. C. S. D.; XAVIER, A; OTONI, W. C. Organogenesis of the leaf explant of Eucalyptus grandis x E-urophylia clones. **Pesquisa Agropecuaria Brasileira**, v. 39, n. 5, p. 421–430, 2004.

AZMI, A.; NOIN, M.; LANDRÉ, P.; et al. High frequency plant regeneration from Eucalyptus globulus Labill. hypocotyls: Ontogenesis and ploidy level of the regenerants. **Plant Cell, Tissue and Organ Culture**, v. 51, p. 9–16, 1997.

BANDYPADHYAY, S.; CANE, K.; RASMUSSEN, G.; HAMILL, J. D. Efficient plant regeneration from seedling explants of two commercially important temperate eucalypt species Eucalyptus nitens and Eucalyptus globulus. **Plant Science,** v. 140, n. 2, p. 189–198, 1999.

BARONE, A.; CHIUSANO, M. L.; ERCOLANO, M. R.; et al. Review Article Structural and Functional Genomics of Tomato. **Hindawi Publishing Corporation International Journal of Plant Genomics**, p. 12, 2008.

BETTENCOURT, G. M. DE F.; SOCCOL, C. R.; GIOVANELLA, T. S.; et al. **Agrobacterium-mediated transformation of two elite clones of Eucalyptus urophylla.** *In preparation,* 2015.

BRANDALISE, M. Isolamento e caracterização de promotores

tecidoespecíficos de raiz e folha de *Coffea arabica***. (Tese) Doutorado em Genética - Instituto de Biociências**, Universidade Estadual Paulista, p. 140, 2007.

BRITT, A. B.; MAY, G. D. Re-engineering plant gene targeting. **Trends in plant science**, v. 8, n. 2, p. 90–5, 2003.

BRONDANI, G. E.; WENDLING, I.; BRONDANI, A. E.; et al. Dynamics of adventitious rooting in mini-cuttings of *Eucalyptus benthamii* x *Eucalyptus dunnii.* **Acta Scientiarum. Agronomy**, v. 34, n. 2, p. 169–178, 2012.

CHITRA, D. S. V.; PADMAJA, G. Shoot regeneration via direct organogenesis from in vitro derived leaves of mulberry using thidiazuron and 6-benzylaminopurine. **Scientia Horticulturae**, v. 106, p. 593–602, 2005.

CID, L. P. B.; GOMES, A. C. M.; COSTA, S. B. R.; BRASILEIRO, A. C. M. Plant regeneration from seedling explants of Eucalyptus grandis X E. urophylla. **Plant Cell Tissue and Organ Culture**, v. 56, p. 17–23, 1999.

CLOSE, D.; BEADLE, C.; MCARTHUR, C. **Understanding and manipulating stress physiology of eucalypt seedlings to improve survival and growth : Preliminary report**. Tasmania, 2003.

COSTA, C. DOS S. Caracterização de promotores de Eucalipto com expressão tecido-específica: raiz e folha. **(Dissertação) Mestrado em Genética, Instituto de Biociências,** Universidade Estadual Paulista. 2011.

DIALLO, N.; DUHOUX, E. Organogenèse et Multiplication «in vitro» chez l'Eucalyptus camaldulensis. **Journal of Plant Physiology**, v. 115, n. 3, p. 177–182, 1984.

DIBAX, R.; DESCHAMPS, C.; BESPALHOK FILHO, J. C.; et al. Organogenesis and Agrobacterium tumefacienes-mediated transforation of *Eucalypruts saligna* with P5CS gene. **Biologia Plantarum**, v. 54, n. 1, p. 6–12, 2010a.

DIBAX, R.; EISFELD, C. D. L.; CUQUEL, F. L.; KOEHLER, H. PLANT REGENERATION FROM COTYLEDONARY EXPLANTS OF Eucalyptus camaldulensis. **Scientia Agricola**, v. 62, n. 4, p. 406–412, 2005.

DIBAX, R.; QUISEN, R. C.; BONA, C.; QUOIRIN, M. Plant Regeneration from Cotyledonary Explants of *Eucalyptus camaldulensis* Dehn and Histological Study of Organogenesis in Vitro. **Braz. Arch. Biol. Technol.**, v. 53, n. 2, p. 311–318, 2010b.

ELISSETCHE, J. P.; VALENZUELA, S.; GARCÍA, R.; et al. Transcript abundance of enzymes involved in lignin biosynthesis of Eucalyptus globulus genotypes with contrasting levels of pulp yield and wood density. **Tree Genetics & Genomes**, v. 7, n. 4, p. 697–705, 2011.

EZHOVA, T. A. Genetic Control of Totipotency of Plant Cells in an in vitro Culture. **Russian Journal of Developmental Biology**, v. 34, n. 4, p. 197–204, 2003.

FERREIRA, M. E.; GRATTAPAGLIA, D. **Introdução ao uso de marcadores moleculares em análise genética.** 3rd ed. Brasília: EMBRAPA-CERNAGEM Documento 20, 1998.

GEORGE, E. F.; HALL, M. A.; KLERK, G.-J. DE. **Plant Propagation by Tissue Cutlure 3rd Edition Vol 1. The Background**. 2008.

GLOCKE, P.; COLLINS, G.; SEDGLEY, M. 6-Benzylamino purine stimulates in vitro shoot organogenesis in Eucalyptus erythronema, E. stricklandii and their interspecific hybrids. **Scientia Horticulturae**, v. 109, p. 339–344, 2006.

GONZÁLEZ, E. R. Transformação genética de *Eucalyptus grandis* e do híbrido *E . grandis x E . urophylla* via *Agrobacterium.* **Tese (Doutorado) Escola Superior de Agricultura "Luis de Queiroz"-** Universidade de São Paulo. p. 93, 2002.

HAJARI, E.; WATT, M. P.; MYCOCK, D. J.; MCALISTER, B. Plant regeneration from induced callus of improved Eucalyptus clones. **South African Journal of Botany**, v. 72, p. 195 – 201, 2006.

HERVÉ, P.; JAUNEAU, A.; PÂQUES, M.; et al. A procedure for shoot organogenesis in vitro from leaves and nodes of an elite Eucalyptus gunnii clone: comparative histology. **Plant science**, v. 161, p. 645–653, 2001.

HO, C.-K.; CHANG, S.-H.; TSAY, J.-Y.; et al. Agrobacterium tumefaciens -mediated transformation of Eucalyptus camaldulensis and production of transgenic plants. **Plant Cell Reports**, v. 17, n. 9, p. 675–680, 1998. Disponível em: <http://link.springer.com/10.1007/s002990050464>. .

HUETTEMAN, C. A.; PREECE, J. E. Thidiazuron: a potent cytokinin for woody plant tissue culture . **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 33, p. 105–119, 1993.

IBÁ. O Relatório Ibá 2015. Disponível em: <http://www.iba.org/images/shared/iba_2015.pdf>. Acesso em: 26/10/2015.

JEFFERSON, R. A. EXPERIMENTAL PROTOCOLS: Assaying Chimeric Genes in Plants: The *GUS* Gene Fusion System. **Plant Molecular Biology Reporter**, v. 5, n. i, p. 387–405, 1987.

LEVA, A. R.; PETRUCCELLI, R.; RINALDI, L. M. R. Somaclonal Variation in Tissue Culture: A Case Study with Olive. **Recent Advances in Plant in vitro Culture**. p.123–150, 2012.

LLOYD, G.; MCCOWN, B. Commercially-feasible micropropagation of Mountain laurel, Kalmia latifolia, by use of shoot tip culture. **Int. Plant Prop. Soc. Proc.**, v. 30, p. 421–427, 1981.

MA, C.; DEEPIKA, R.; MYBURG, A. A; RANIK, M.; STRAUSS, S. H. Development of Eucalyptus tissue culture conditions for improved in vitro plant health and transformability. **BMC Proceedings**, v. 5, n. Suppl 7, p. P153, 2011.

MEINKE, D. W.; CHERRY, J. M.; DEAN, C.; ROUNSLEY, S. D.; KOORNNEEF, M. Arabidopsis thaliana: a model plant for genome analysis. **Science.**, v. 282, n. 5389, p. 662–679, 1998.

MENDONÇA, G. E.; STEIN, V. C.; BALIEIRO, F. P.; et al. Genetic transformation of Eucalyptus camaldulensis by Agrobalistic method. **Revista Árvore**, v. 37, n. 3, p. 419–429, 2013.

MIÑANO, H. S.; GONZÁLEZ-BENITO, M. E.; MARTÍN, C. Molecular characterization and analysis of somaclonal variation in chrysanthemum cultivars using RAPD markers. **Scientia Horticulturae**, v. 122, n. 2, p. 238–243, 2009.

MITSUDA, N.; OHME-TAKAGI, M. Functional Analysis of Transcription Factors in Arabidopsis. **Plant Cell Physiol.**, v. 50, n. 7, p. 1232–1248, 2009.

MULLINS, K. V.; LLEWELLYN, D. J.; HARTNEY, V. J.; STRAUSS, S.; DENNIS, E. S. Regeneration and transformation of Eucalyptus camaldulensis. **Plant Cell Reports**, v. 16, n. 11, p. 787–791, 1997.

MUNDHARA, R.; RASHID, A. TDZ-induced triple-response and shoot formation on intact seedlings of Linum, putative role of ethylene in regeneration. **Plant Science**, v. 170, p. 185–190, 2006.

MUNIR, F.; NAQVI, S.; MAHMOOD, T. In vitro culturing and

assessment of somaclonal variation of Solanum tuberosum var . desiree. **Turk J Biochem.**, v. 36, n. 4, p. 296–302, 2011.

MURALIDHARAN, E. M.; MASCARENHAS, A. F. In vitro plantlet formation by organogenesis in Eucalyptus camaldulensis and by somatic embryogenesis in Eucalyptus citriodora. **Plant cell reports**, v. 6, p. 256– 259, 1987.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Phisiol. Plant**, v. 15, p. 473– 497, 1962.

MURTHY, B. N. S.; MURCH, S. J. thidiazuron : a potent regulator of in vitro plant morphogenesis. **In Vitro Cell Dev. Biol.**, v. 34, n. December, p. 267–275, 1998.

MYCOCK, D. J.; WATT, M. P. Shoot and root morphogenesis from Eucalyptus grandis x urophylla callus. **African Journal of Biotechnology**, v. 11, n. 101, p. 16669–16676, 2012. Disponível em: <http://www.academicjournals.org/AJB>. Acesso em: 5/10/2014.

NAVARRO, M.; AYAX, C.; MARTINEZ, Y.; et al. Two EguCBF1 genes overexpressed in Eucalyptus display a different impact on stress tolerance and plant development. **Plant biotechnology journal**, v. 9, n. 1, p. 50–63, 2011. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/20492548>. Acesso em: 5/10/2014.

NDAKIDEMI, C. F.; MNENEY, E.; NDAKIDEMI, ATRICK A. Effects of Ascorbic Acid in Controlling Lethal Browning in in Vitro Culture of Brahylaena huillensis Using Nodal Segments. **American Journal of Plant Sciences**, v. 5, p. 187–191, 2014. Disponível em: <http://dx.doi.org/10.4236/ajps.2014.51024>. Acesso em: 5/10/2015.

NORTH, J. J.; NDAKIDEMI, P. A.; LAUBSCHER, C. P. Effects of antioxidants, plant growth regulators and wounding on phenolic compound excretion during micropropagation of Strelitzia reginae. **International Journal of the Physical Sciences**, v. 7, n. 4, p. 638 – 646, 2012.

OLIVEIRA, C. DE; DEGENHARDT-GOLDBACH, J.; BETTENCOURT, G. M. DE F.; AMANO, E.; QUOIRIN, M. Micropropagation of Eucalyptus grandis x E. urophylla AEC 224 clone. **Journal of F Research**, 2015.

OLIVEIRA-CAUDURO, Y.; ADAMUCHIO, L. G.; DEGENHARDT-GOLDBACH, J.; et al. Organogênese indireta a partir de explantes foliares e multiplicação in vitro de brotações de Eucalyptus benthamii x Eucalyptus dunnii. **Ciência Florestal**, v. 24, n. 2, p. 347–355, 2014.

POKE, F. S.; VAILLANCOURT, R. E.; POTTS, B. M.; REID, J. B. Genomic research in Eucalyptus. **Genetica**, v. 125, n. 1, p. 79–101, 2005. Disponível em: <http://www.ncbi.nlm.nih.gov/pubmed/16175457>. Acesso em: 23/10/2014.

PREECE, J. E.; IMEL, M. R. Plant regeneration from leaf explants of Rhododendron "P.J.M. Hybrids'. **Scientia Horticulturae**, v. 48, p. 159–I70, 1993.

QUOIRIN, M.; LEPOIVRE, P. Etude de Millieux adaptes aux Cultures In Vitro de Prunus. **acta Horticulturae**, v. 78, p. 437–442, 1977.

QUOIRIN, M.; QUISEN, R. Advances in genetic transformation of Eucalyptus species. In: C. Franche (Org.); **Molecular biology of tropical plants**. p.41–56, 2006.

R DEVELOPMENT CORE TEAM. R: A Language and Environment for Statistical Computing. **The R Foundation for Statistical Computing**. Vienna,

Austria.ISBN: 3-900051-07-0. 2011. Available online at http://www.R-project.org/.

RAHIM, F.; JABEEN, M.; ILAHI, I. Mass Propagation in Eucalyptus camaldulensis Dehn. **Asian Journal of Plant Sciences**, v. 2, n. 2, p. 184–187, 2003.

RIBEIRO, C. L. Caracterização molecular de um promotor raiz-específico de Eucalipto. **Universidade Estadual Paulista Júlio de Mesquita Filho**. p. 53 2009.

RODRIGUES, M. I.; BRAVO, J. P.; SASSAKI, F. T.; SEVERINO, F. E.; MAIA, I. G. The tonoplast intrinsic aquaporin (TIP) subfamily of Eucalyptus grandis: Characterization of EgTIP2, a root-specific and osmotic stress-responsive gene. **Plant science : an international journal of experimental plant biology**, v. 213, p. 106–13, 2013.

RUŽIĆ, D. V.; VUJOVIĆ, T. I. The effects of cytokinin types and their concentration on in vitro multiplication of sweet cherry cv. Lapins (Prunus avium L.). **Hort. Sci. (Prague)**, v. 35, n. 1, p. 12–21, 2008.

SAAD, A. I. M.; ELSHAHED, A. M. Plant Tissue Culture Media. In: A. Leva; L. M. R. Rinaldi (Orgs.); **Recent Advances in Plant in vitro Culture**. p.220, 2012. InTech.

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS, T.; SPRING, H. L. C. **Molecular Cloning: A Laboratory Manual.** 2nd ed. ed. Colc Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press, 1989.

SERRANO, L.; ROCHANGE, F.; SEMBLAT, J. P.; et al. Genetic transformation of Eucalyptus globulus through biolistics : complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. **Journal of experimental botany**, v. 47, n. 295, p. 285–290, 1996.

SHIMAMOTO, K.; KYOZUKA, J. Rice as a model for comparative genomics of plants. **Annu. Rev. Plant Biol.**, v. 53, p. 399–419, 2002.

SILVA, A. L. L. DA; OLIVEIRA, Y. DE; COSTA, J. DA L.; et al. Shoot tip and cotyledon explants of Eucalyptus saligna Sm. cultivated on different kanamycin levels. **Journal of Biotechnology and Biodiversity**, v. 1, n. 1, p. 1–5, 2010.

SUN, S.; ZHONG, J.; LI, S.; WANG, X. Tissue culture-induced somaclonal variation of decreased pollen viability in torenia (Torenia fournieri Lind .). **Botanical Studies**, v. 54, n. 36, p. 1–7, 2013.

SUTTLE, J. C. Disruption of the Polar Auxin Transport System in Cotton Seedlings following Treatment with the Defoliant Thidiazuron. **Plant Physiol**, v. 86, n. 1, p. 241–245., 1988.

TAYLOR, G. Populus: Arabidopsis for forestry. Do we need a model tree? **Annals of Botany**, v. 90, p. 681–689, 2002.

TIBOK, A.; BLACKHALL, N. W.; POWER, J. B.; DAVEY, M. R. Optimized plant regeneration from callus derived from seedling hypocotyls of Eucalyptus urophylla. **Plant Science**, v. 110, p. 139–145, 1995.

TOURNIER, V.; GRAT, S.; MARQUE, C.; et al. An efficient procedure to stably introduce genes into an economically important pulp tree (Eucalyptus grandis × Eucalyptus urophylla). **Transdenic Research**, v. 12, p. 403–411, 2003.

TSURO, M.; KODA, M.; INOUE, M. Comparative effect of different types of cytokinin for shoot formation and plant regeneration in leaf-derived callus of

lavender (Lavandula vera DC). **Scientia Horticulturae**, v. 81, p. 331–336, 1999.

VAUGHAN, S. P.; JAMES, D. V.; LINDSEY, K.]; MASSIAH, A. J. Characterization of FaRB7, a near root-specific gene from strawberry (Fragaria x ananassa Duch.) and promoter activity analysis in homologous and heterologous hosts. **Journal of Experimental Botany**, v. 57, n. 14, p. 3901–3910, 2006.

VERA BRAVO, C. D. **Controle genético e histogênese da regeneração de progênies de Eucalyptus grandis in vitro.**, 2005.

WOLFF, K.; PETERS-VAN RIJN, J. Rapid detection of genetic variability in chrysanthemum (Dendrathema grandiflora Tzevlev) using random primers. **Heredity**, v. 71, p. 335–341, 1993.

YADAV, S. K.; KATIKALA, S.; YELLISETTY, V.; et al. Optimization of Agrobacterium mediated genetic transformation of cotyledonary node explants of Vigna radiata. **SpringerPlus**, v. 1, n. 59, p. 8, 2012.

CHAPTER II- *Agrobacterium*-mediated transformation of an elite clone of *Eucalyptus urophylla*

ABSTRACT

This study aimed to develop an efficient *Agrobacterium*-mediated transformation protocol of the elite clone BRS07-01 of *Eucalyptus urophylla*. First, the effect of Kanamycin and Geneticin were evaluated on the organogenesis from leaves. Afterwards, several experiments were carried out to evaluate different features of genetic transformation. Pre and co-culture times, concentrations of Acetosyringone (AS), sonication time with leaves and microshoots and antibiotics concentrations after transformation were evaluated. Putative transformants were confirmed histochemical GUS expression and the insertion of *uidA* and *nptII* genes in the genome via PCR. Using leaves explants we observed up to 2.67% of transformation efficiency (TE) with 50 µM AS on liquid co-culture + 100 µM AS on solid co-culture. However, up to 20.83% of TE was observed with microshoot explants submitted to 2 min of sonication and co-cultured with 100 µM AS and cultured with 150 mgL^{-1} of Kanamycin. We developed a highly efficient *Agrobacterium*-mediated transformation protocol for clone BRS07-01 of *Eucalyptus urophylla* using microshoots as explants.

Keywords: *gus*, npt*II*, genetic transformation, Acetosyringone, sonication.

1. INTRODUCTION

Eucalyptus is a major source of hardwood globally. This genus exhibits high adaptability and phenotypic plasticity and therefore is extensively grown worldwide (GRATTAPAGLIA *et al.*, 2012). In Brazil, *E. grandis, E. urophylla* and their hybrids are the most commonly cultivated species. *E. urophylla* has an ability to grow on poor soils, tolerance to low rainfall and resistance to pests and diseases (GHERARDI HEIN, 2011).

Breeding programs of economically important tree species target mainly the productivity. Brazil, in particular, has a governmental breeding program for *Eucalyptus* at Embrapa Forestry, the federal company for agricultural research. However, the relatively long reproductive cycles and genetic complexity turns genetic breeding and large-scale cloning relatively difficult (NEHRA *et al.*, 2005). In this context, a significant amount of research has been geared towards improving genetic transformation protocols of *Eucalyptus* species to make them efficient and able to be used as important tools for conventional breeding programs (NEHRA *et al.*, 2005; GIRIJASHANKAR, 2011).

So far, genetic transformation of commercial forestry species has targeted on growth rate, wood quality, lignin content, stress tolerance (both biotic and abiotic) and production of pharmaceutical products, among others (TOURNIER *et al.*, 2003; HAJARI *et al.*, 2006; WALTER and MENZIES, 2010; CASTELLANOS-HERNÁNDEZ *et al.*, 2011; RIBEIRO, 2012).

Genetic transformation consists of three main steps: (1) infection or gene insertion, (2) selection, and (3) *in vitro* regeneration of the transgenic plants. Gene insertion can be direct or indirect. On the latter, *Agrobacterium*-mediated transfer is the most common method. To enable effective selection of transformed plants, the transgene is joined to a selective gene. The selective gene may code for a substrate that can be identified on certain media, microscopic screening or impart selective resistance (PENNA *et al.*, 2002) to antibiotics or herbicides. To date, the neomycin phosphotransferase gene (*nptII*), which encodes an enzyme that inactivates different antibiotics of amino-glycosylated group such as Kanamicyns (Km) A, B and C, Geneticin (G-418), Neomycin and others (QUISEN *et al.*, 2009; YU *et al.* 2003) is the most used gene for selecting transgenic on selective growth media.

The first *Agrobacterium*-mediated transformation of *Eucalyptus* species was reported over two decades ago (CHRIQUI *et al.*, 1992). However, due to regulatory constraints, transgenic trees are only just starting to get license for cultivation in Brazil (CTNBIO, 2015). The challenges for commercial programs is the development of high efficiency transformation and regeneration protocols, especially for recalcitrant *Eucalyptus* species (DE LA TORRE *et al.*, 2014; MATSUNAGA *et al.*, 2012; RIBEIRO, 2012). The transformation efficiency of *Eucalyptus* genetic transformation are in the most cases still very low: 1.16-

2.33% for *E. tereticornins* (AGGARWAL *et al.*, 2011), 1.46% for *E. urograndis* (BALIEIRO, 2013), 0.5% for *E. saligna* (DIBAX *et al.*, 2010), an average of 3.98% (MATSUNAGA *et al.*, 2012) and 1.2% (MORALEJO *et al.*, 1998) for *E. globulus.* However, recently, de la Torre et. al. (2014) reported a protocol with an efficiency of over 65% of positive GUS expression for *E. globulus* clone.

This study aimed to develop an efficient genetic transformation protocol for *E. urophylla* clone BRS07-01.

2. MATERIAL AND METHODS

2.1. Plant material

Leaves of the clone BRS07-01 of *E. urophylla* were used as explants. The micropropagated clone was maintained under micropropagation on MS medium (MURASHIGE and SKOOG, 1962) containing 30 g L^{-1} sucrose, 0.88 µM BAP, 0.2 mg L^{-1} myoinositol and 7 gL^{-1} of agar, in a growth room at 23 ±2°C under white fluorescent light with an irradiance of 40 $\mu mol.m^{-2}.s^{-1}$ and 16 hours of photoperiod. This clone was selected in the breeding program of Embrapa Forestry, chosen for is fast growth (wood volume of 0,047 m^3/tree at the 21st month) and is suitable for multiple uses (Estefano Paludzyszyn Filho, Embrapa Forestry researcher, personal communication). In a previous work, we developed an protocol which allowed 85% regeneration via organogenesis for this clone.

2.2. In vitro organogenesis

BRS07-01 leaves were collected and inoculated in petri dishes with 20 mL of callus induction medium (CIM): WPM medium salts (LLOYD and MCCOWN, 1981) containing 30 gL^{-1} sucrose, 0.5 µM TDZ, 0.1 µM NAA, 0,1 mgL^{-1} myoinositol, 500 mg L^{-1} PVP and 7 gL^{-1} agar, for 28 days at 23 ± 2°C in darkness. Following that, they were transferred to shoot induction medium (SIM): WPM containing 30 gL^{-1} sucrose, 5.0 µM BAP, 0.5 µM NAA, 0,1 mg L^{-1} myo-inositol, 500 mgL^{-1} PVP and 7 gL^{-1} agar, for 60 days in a growth room at 23±2°C with 16h of photoperiod (light irradiance of 40 $\mu mol.m^{-2}.s^{-1}$). The

explants were transferred to fresh medium every 14 days. The pH of all media was adjusted to 5.8 before autoclaving for 20 min at 120°C 1atm.

2.3. Effect of Kanamycin and Geneticin as selective agents on organogenesis of clone BRS07-0

In order to evaluate two antibiotics and their doses on organogenesis of clone BRS07-01, the explants were cultivated on the basal CIM and SIM containing the antibiotics Kanamycin (Km) (0, 12.5, 25, 50, 75, 100 and 125 mg L^{-1}) or Geneticin (Gen) (0, 2, 3, 4, 5, 6, 7 and 8 mg L^{-1}). Antibiotic solutions were sterilized by filtration with a 0.22 µm filter membrane and added to the medium after autoclaving.

2.4. Experimental design

The experimental designs were completely randomized and each treatment consisted of 2 replicates, with 40 explants per Petri dish and was repeated twice. After 30 and 90 days oxidation, presence of anthocyanin, callus induction, shoot formation in the explants with callus and number of shoots per explants (the last one only after 90 days) were evaluated. For the genetic transformation experiments, each treatment consisted on 150 explants, with 30 explants per petri dish, and the experiments were repeated twice.

2.5. Genetic transformation of clone BRS07-01

Basal transformation system: leaves were used as explants and were cultured as described above. To improve the rates of infection and subsequent transformation, small transversal cuts were made on explant leaves. For bacterial infection, the explants were immersed in a bacteria suspension (described below) for 30 min at 120 rpm at 28°C. Explants were then dried in sterile filter paper and cultured on CIM media for 3 days in the dark. To kill the *Agrobacterium*, explants were washed on sterile distilled water with 200 mgL^{-1} cefotaxime. Following that, they were transferred to CIM media supplemented with 200 mgL^{-1} amoxicillin and 25 mgL^{-1} Km as the selective agent. For callus induction, explants were cultured in the dark at 23 ± 2ºC for 28 days and were then transferred to a growth room at 23 ±2°C and 16 hours of photoperiod (at 40

µmol.m^{-2}.s^{-1}). The Km concentration was increased after 30 days to 50 mgL^{-1} and after the next subculture of 14 days to 75 mgL^{-1}, and subsequently to 100 mgL^{-1} of Km, until the end of the experiment.

Bacteria suspension: the *Agrobacterium tumefaciens* strain EH105 harboring pCAMBIA 2301 vector (figure 1), harboring *nptII* selection gene and *uidA* gene, both under the control of the CaMV35S promotor, was grown overnight in a LB medium (10.0 gL^{-1} Tryptone, 10.0 gL^{-1} NaCl and 5.0 gL^{-1} yeast extract, pH= 7.0) Sambrook et al. (1989) media supplemented with 50 mgL^{-1} of Km at 28°C and 120 rpm until OD_{600nm} 0.6-1.0. The bacteria culture was then centrifuged for 10 min at 7500 rpm (IEC Multi RF, Thermo Electron co., USA), and the pellet suspended on ½ MS liquid medium (pH 5.8)].

The following experiments were carried out to improve the genetic transformation efficiency:

Experiment I: pre-culture period (0, 1, 2, 3 or 4 days), in which the explants were cultured on CIM as described above;

Experiment II: co-culture period (2, 3, 4 or 5 days). After infection, the explants were cultured on evaluated days on CIM;

Experiment III: Acetosyringone concentrations (0, 50, 100, 200 or 400 µM) added on pre-culture (1 day) and/or co-culture (3 days): T1: LC 0 µM AS + SC 0 µM AS; T2: LC 50 µM + SL 0 µM AS; T3: LC 0 µM AS + SC 100 µM AS; T4: LC 50 µM AS + SC 100 µM AS; T5: LC 100 µM AS + SC 0 µM AS; T6: LC 100 µM AS + 100 µM AS; T7: LC 200 µM AS + SC 200 µM AS; T8: LC 200 µM AS + SC 0 µM AS; T9: LC 0 µM AS + SC 400 µM AS; T10: LC 400 µM AS + SC 0 µM AS and T11: LC 400 µM AS + SC 400 µM AS. (LC – liquid co-culture, SC-solid co-culture, AS- acetosyringone).

Experiment IV: to compare types of explant we tested leaves and micro-shoots (0.5-2 cm) with at least 2 or 3 leaves as described by Gallego *et al.* (2003), with some modifications. The explants were pre-cultured on CIM for 1 day and then immersed in bacteria suspension and submitted to sonication treatment (40kHz, Ultra Cleaner 1400A, Unique®) for 60, 90 or 120 seconds. Explants were then co-cultured on CIM media with 100 µM of AS for 3 days in the dark, washed on sterile distilled water with 200 mgL^{-1} cefotaxime, and

placed on selective medium. The microshoots were cultured on MS media containing 30 g L^{-1} sucrose, 0.88 µM BAP, 0.2 mg L^{-1} myo-inositol, and 7 gL^{-1} of agar supplemented with 200 mgL^{-1} of amoxicillin and 150 mgL^{-1} of Km in a growth room at 23 ±2°C and 16hrs of photoperiod. The leaves explants were cultured as explained on the Basal transformation system above.

Experiment V: Km concentrations (25 or 50 mgL^{-1}) and 5 mgL^{-1} of Gen. The selective concentrations were kept constant for the first 30 days and afterward in the treatments with Km the concentration was increased up to 100 mgL^{-1} and in the treatment with Gen the concentration was increased up to 15 mgL^{-1}.

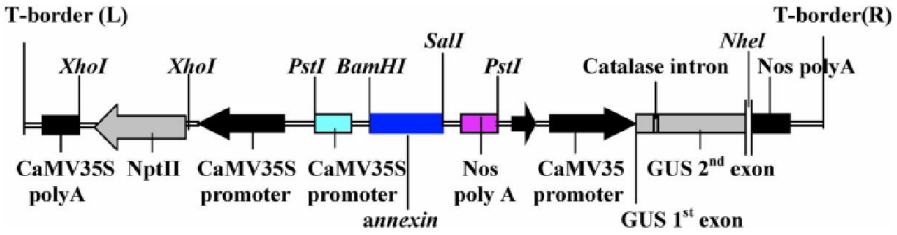

Figure 1. pCAMBIA 2301 vector scheme. Source: YADAV *et al.*, (2012).

Gene insertion and screening of transformants

Histochemical and PCR analyses were carried out to confirm the expression of the transgene.

Histochemical analysis: Explants were immersed in a solution containing 10mM $Na_2EDTA.H2O$, 0,1% Triton X-100, 0,1 M NaH_2PO_4, 0,5 M $K_3Fe(CN)_6$, 250 µg.mL-1 5-bromo-4- chloro-3-indolyl β-D-glucuronic acid (X-GLUC, Gold Biotecnhology Inc, USA) with 20% of methanol and incubated for 16 h at 37°C (JEFFERSON, 1987). Following that, the explants were washed several times with 70% ethanol to remove chlorophyll. The material was observed for GUS gene expression by the blue staining.

PCR Analysis: DNA samples from putative transgenic explants as well as the control explant was extracted using the CTAB 2% (Catonic Hexadecyl Trimethyl Ammonium Bromide) protocol according to Ferreira; Grattapaglia (1998). The PCR was performed using the forward and reverse primes of *uidA* (F: 5´-CAGCGCGAAGTCTTTATACCG-3´; R: 5´-

ATGCGTCACCACGGTGATATCG-3´) and *nptII* genes (F: 5´-TCGGCTATGACTGGGCACAACAGA-3´; R: 5´-AAGAAGGCGATAGAAGGCGATGCG-3´). The PCR mixture (25 µl) contained 1.0 U taq DNA polymerase, 2.0 mM $MgCl_2$, 1x PCR buffer, 2.0mM of each dNTP, 0.4 mM of each forward and reverse primer (Invitrogen, Br) and approximately 50ng template DNA. For positive control 4.0ng of pCAMBIA 2301 plasmid DNA was used. The PCR was performed using a Veriti 96 Well Thermal cycler from Applied Biosystem.

PCR programs for the genes:

nptII: 1 cycle at 94°C for 5min, 30 cycles at 94°C for 1min, 65°C for 1min and 72°C for 2 min and 1 cycle at 72 °C for 7 min.

uidA: cycle at 94°C for 4 min, 35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 1 min, and 1 cycle at 72°C for 7 min.

Amplification products were separated on 1% agarose gel electrophoresis with ethidium bromide solution (0.5 mg/ml, Invitrogen, Br) in 1x buffer TBE (0.04 M TRIS-acetate, 1 mM EDTA, pH= 8) at 80 V for 1 hr. The gel was visualized and photographed using a Cannon PowerShot S2 IS camera under Syngene Bio Imaging program.

2.6. Experimental design

Experiments using leaves as explants were carried out with 150 explants and the experiment with microshoot (experiment IV), was used flasks with 5 explants, totalizing 50 explants for each treatment. The experiments were repeated twice. The genetic transformation efficiency were evaluated after 120 days for leaves and 60 days for microshoots.

2.7. Statistical analyses

Statistical analyses were performed for the experiments evaluating the selective agents only. An analysis of variance (ANOVA) was performed on the results. The binary data adjustment was done via binomial distribution probability; that for quantitative data was via the Poisson distribution. A logistic regression analysis was also performed on the binary data. For treatments with qualitative factors, the Tukey test 5% was used to compare treatments.

3. RESULTS

3.1. Effect of Kanamycin as selective agent

This experiment aimed to establish the minimal lethal dose of Km as selective agent on the organogenesis of clone BRS07-01. After 30 and 90 days on Km, the variance analysis showed that all variables had a significant effect on the treatments (data not shown).

The regression curves were calculated for callus and shoot formation, oxidation and anthocyanin, after 30 and 90 days on culture (Figure 2). After 30 days, callus formation was 3-fold higher in the control (74.4%) than on 75 mgL^{-1} Km. Shoot formation reached up to 25% in the control after 30 days, and was 12-fold higher on explants cultivated on 12.5 mgL^{-1} Km when compared to explants on 75 mgL^{-1} Km (3.2%). Oxidation frequency was also statistically different among treatments, and ranged from 81.9% in the control to 100% in explants on 100 mgL^{-1} and 125 mgL^{-1} Km.

After 90 days on culture, callus formation was 5-fold higher on explants cultured on 12.5 mgL^{-1} Km (65.5%) when compared to explants cultured on 100 mgL^{-1} Km. In the control, 78.1% of the explants formed callus. The shoot formation was 38-fold higher on 12.5 mgL^{-1} Km (32.8%) when compared to 75 mgL^{-1} Km (3.2%). Explants with anthocyanin rate ranged from 10.1% in the explants cultured on 75 Km to 27.7% in the explants on 12.5 mgL^{-1} Km. Explants on 100 mgL^{-1} and 125 mgL^{-1} Km were all oxidized and did not show anthocyanin. Oxidation was 3-fold higher on 25 mgL^{-1} Km than on 12.5 mgL^{-1} Km (Figure 3).

After 30 days, most of the explants cultured on media with concentrations of 100 mgL^{-1} or 125 mgL^{-1} Km turned brown or pale and died.

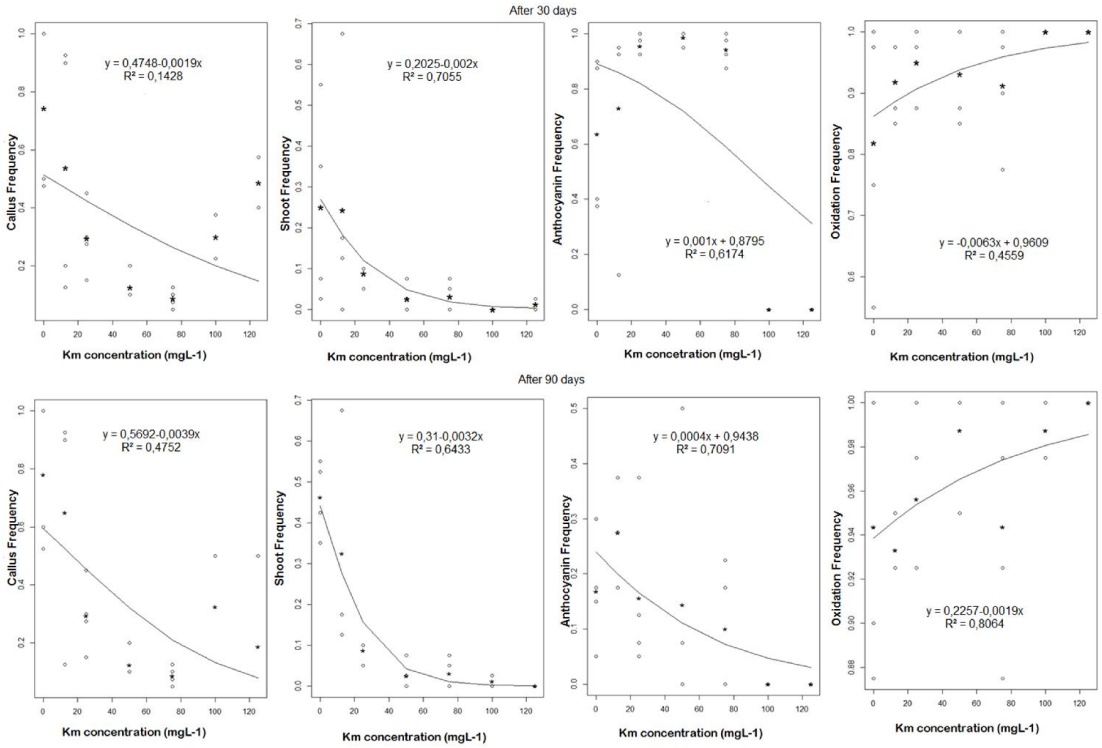

Figure 2. Logistic regression analysis and adjusted curves data of different Kanamycin concentrations (12.5, 25, 50, 75, 100 and 125 mgL^{-1}) and a control without Km effect on organogenesis of clone BRS07-01 of *Eucalyptus urophylla*. Evaluations after 30 and 90 days of cultured.

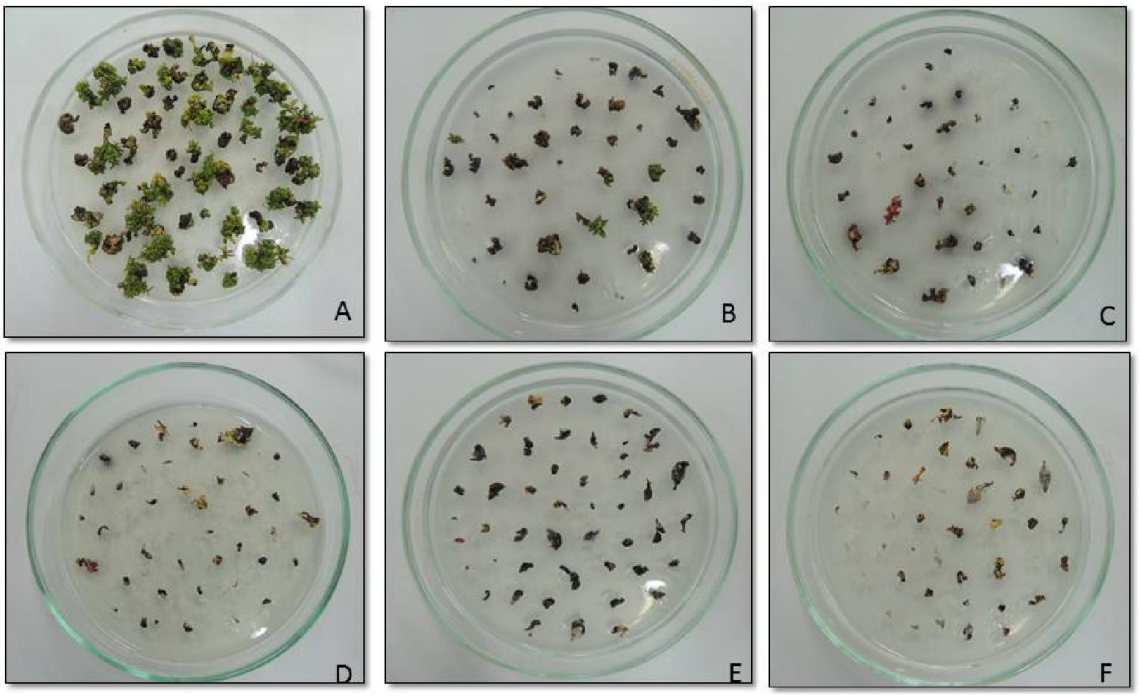

Figure 3. Shoots derived from explants cultured for 90 days on Km containing media. (A) Control Km-free media, (B) 12.5 mgL^{-1} Km, (C) 25 mgL^{-1} Km, (D) 50 mgL^{-1} Km, (E) 75 mgL^{-1} Km and (F) 100 mgL^{-1} Km.

3.2. Effect of Geneticin as selective agent

Geneticin was tested at lower concentrations, when compared to kanamycin. Eight concentrations (0, 1, 2, 3, 4, 5, 6, 7 and 8 $mgL\text{-}^{1}$) were evaluated to establish the minimal lethal dose of Gen as selective agent in the organogenesis of clone BRS07-01. Except for shoot induction and anthocyanin after 90 days, the results showed statistical differences for all the variables (Figure 4).

The logistic regression showed that after 30 days, the callus formation was 2.5-fold higher in the control when compared to 7 mgL^{-1} Gen (67.5%). The lower callus formation rate was observed on 8 mgL^{-1} Gen (40.1%). Shoot formation was 12 times higher on explants cultured without Gen than those cultured on 8 mgL^{-1}. The oxidation rate ranged from 37.5% in the control up to 97.5% in the explants cultured on 4 mgL^{-1} Gen. Explants growing on 5 mgL^{-1} Gen have 2-fold higher chances to oxidize than those cultured on the control.

Anthocyanin was observed in all treatments and ranged from 0.12% on 7 mgL^{-1} and 8 mgL^{-1} Gen to 43.8% on 2 mgL^{-1} Gen.

After 90 days on culture, callus formation was observed in 100% of the explants on the control medium (Figure 4). Explants cultured on 2 mgL^{-1} Gen had 2-fold higher chances to form callus than explants cultured on 7 mgL^{-1} Gen. Shoot formation rates were not different among treatments. Explants with callus forming shoots ranged from 45% in the control to 22.9% in 5 mgL^{-1} Gen. With 8 mgL^{-1} Gen shoots were formed on 26.9% of the explants forming callus. The oxidation ranged from 50.5% on 8 mgL^{-1} Gen to 100% on 2, 3, 4 and 5 mgL^{-1} Gen and was statistically different among the treatments. Anthocyanin was also not statistically different and ranged from 21.5% of the explants on 8 mgL^{-1} Gen to 46.2% of the explants on 2 mgL^{-1} Gen (Figure 5).

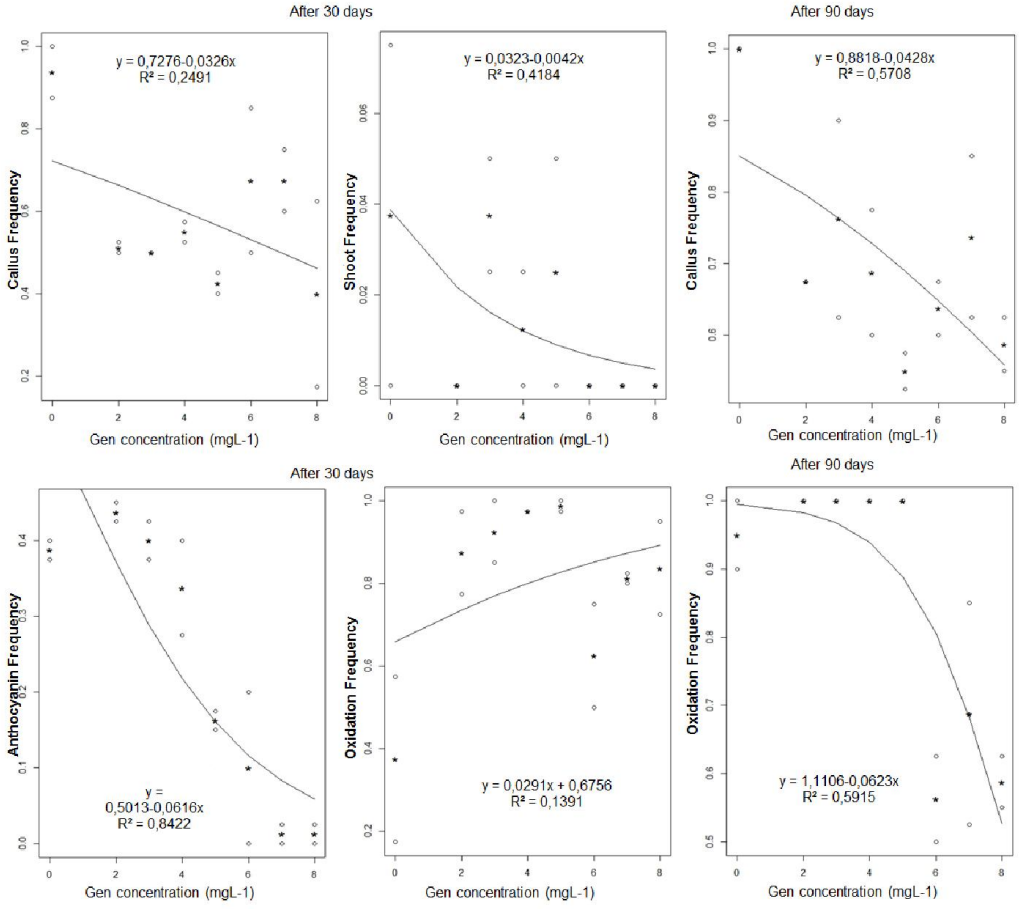

Figure 4. Logistic regression analysis and adjusted curves of the resuming the data of the effect of 8 different concentrations of geneticin (0, 2, 3, 4, 5, 6, 7 and 8 mgL^{-1}) in indirect organogenesis of clone BRS07-01 of *Eucalyptus urophylla*. Evaluations after 30 and 90 days.

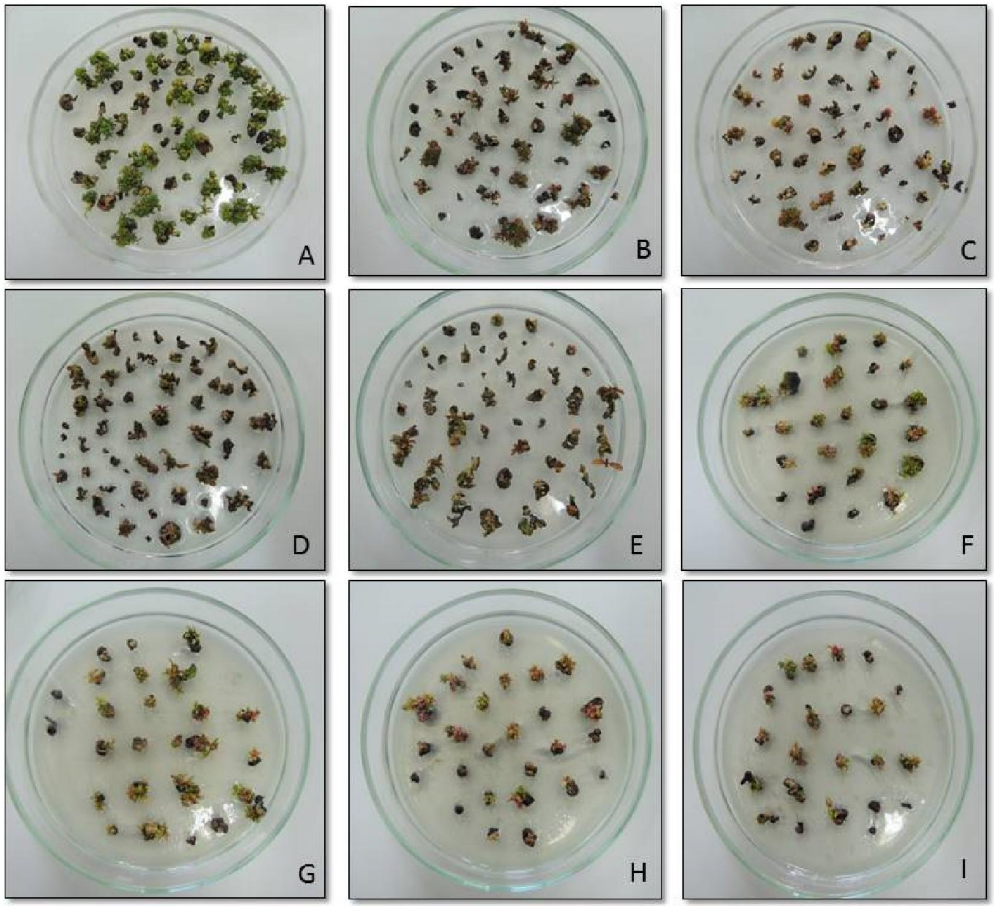

Figure 5. Leave explants cultivated on media containing Gen at 8 different concentrations after 90 days of culture. (A) Control media, (B and C) explants cultured on 2 mgL^{-1} of Gen, (D-E) explants cultured on 3 and 4 mgL^{-1} of Gen, (F-I) survived explants showing shoots tip cultured on 5, 6, 7 and 8 mgL^{-1} of Gen, respectively.

3.3. Genetic transformation

We have tested several factors to increase the genetic transformation efficiency of our clone: different pre-culture days, co-culture days, concentrations of AS on pre and co-culture, sonication before solid co-culture, Km and Gen concentrations and microshoots as explants. Twenty five milligrams per liter of Km were used as the standard concentration for all the experiments, except when selective concentrations were tested.

In the majority of the experiments we were not able to regenerate transgenic plants, and observed high percentages of escapes on kanamycin at low concentrations. The use of AS and Km concentration starting from 50 mgL^{-1}

proved to be essential for the regeneration of transgenic plants of clone BRS07-01.

In the experiments I and II, we have tested the effects of days in pre-culture and co-culture in the transformation efficiency. However, although we observed some shoots growing on 25 Km, all of them proved to be escapes (8.13 and 2.66 %, respectively) with no blue staining to confirm GUS expression and either no amplification by PCR.

Acetosyringone had a positive effect on transformation efficiency on our clone. We observed putative shoots growing at all treatments, except on T5. After *gus* staining and PCR analysis, the higher TE was observed on T4 (LC 50 µM AS + SC 100 µM AS) with 2.67%, followed by 2.0% TE on T9, 1.33% TE on T3, and 0.67% TE on T6 and T7 (Table 1). No amplification for *uidA* and *nptII* genes were observed on the putative shoots growing on T1, 2, 5, 8, 10 and 11, proven to be all escapes. However, all the positive treatments had AS during solid co-culture, independently of concentration, which shows us that the presence of AS is essential to increase *Agrobacterium*-mediated transformation, principally during the co-culture step.

In experiment IV, we tested sonication time before co-culture on leaves or microshoots. Even that had some putative shoots growing on selective media, came from leaves explants from the three treatments, all of them were confirmed as escapes, after *gus* staining and PCR analysis. On the other hand, when microshoots were used as explants, we observed up to 20.83% of TE when the explants were submitted to 120 seconds of sonication. PCR analysis confirmed the genes insertion (Fig 6). On 60 and 90 seconds of sonication, we did not observe any regeneration on media containing 150 mgL^{-1} Km.

In the experiment V we tested two selective agents in the transformation of clone BRS07-01. Four, 2 and 10 putative shoots resulted from the treatments with 25 and 50 mgL^{-1} of Km and 5 mgL^{-1} of Gen, respectively. But only the explants cultured on 50 mgL^{-1} of Km were confirmed as transgenic by histochemical GUS assay and PCR analysis, corresponding to 1.3% of TE. The others antibiotic concentrations evaluated were ineffective for selection and allowed only the growth of escapes.

Table 1. Transformation efficiency from Clone BRS07-01 of *E. uropylla* genetic transformation in presence of Acetosyringone (AS) on liquid and solid co-culture. Transformation efficiency is the ratio of transgenic plant numbers to the total number of explants per treatment. The values does not differ by Tukey ($p<0.05$).

Treatment	Liquid co-culture with AS (µM)	Solid co-culture with AS (µM)	Transformation efficiency
1	0	0	0±0
2	50	0	0±0
3	0	100	1.33±1.8
4	50	100	2.7±2.6
5	100	0	0±0
6	100	100	0.67±1.3
7	200	200	0.67±1.3
8	200	0	0±0
9	0	400	2.0±2.3
10	400	0	0±0
11	400	400	0±0

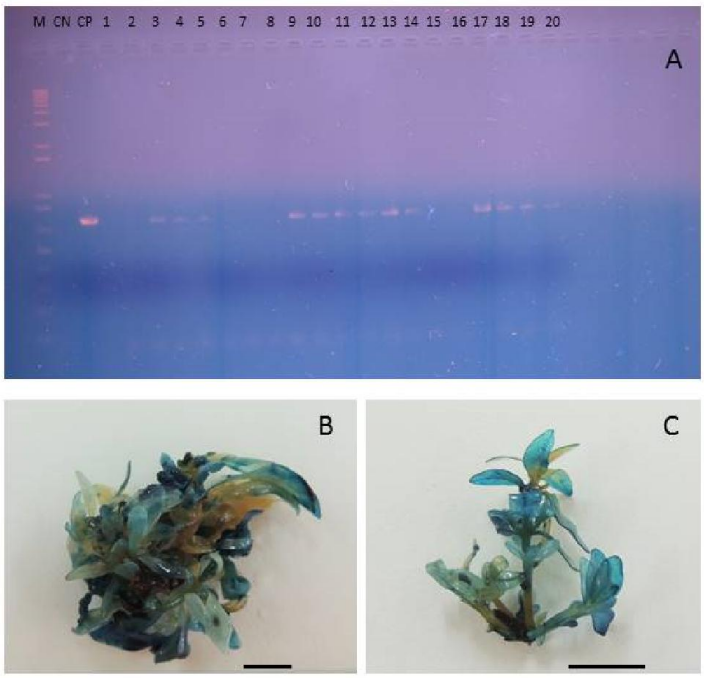

Figure 6. (A) *nptII* PCR amplification from transformants events of Clone BRS07-01. Legend: M- 1kb marker, B- Blank, CP- Positive control, CN- Negative control, 1-20 transformed explants showing *nptII* amplification (≈ 760 bp). (B and C) Stable GUS expression on shoot tip derived from *Agrobacterium*-mediated genetic transformation of clone BRS07-01. Bar = 0.5cm.

4. DISCUSSION

4.1. Selective agent system for clone BRS07-01

We have chosen to work with clone BRS07-01 of *E. urophylla,* once this clone has been selected in the genetic breeding program of Embrapa Forestry. The clone has characteristically fast growth (wood volume of 0,047 m^3/tree at the 21st month) and is suitable for multiple uses (Estefano Paludzyszyn Filho, Embrapa Forestry researcher, personal communication). However, it is not tolerant to drought. As one of the aims of the genetic program is to grow the materials on marginal areas, genetic transformation could be a tool to insert genes which confer tolerance to water stress. For this reason, we needed to develop an efficient transformation protocol for clone BRS07-01.

The appropriate selective agent concentration is the critical point before genetic transformation procedures. There is a thin line between the phytotoxic and harmful effect, affecting the growth of plant cells and the regeneration process (QUISEN *et al.*, 2009). Too high concentrations may kill the non-transformed cells, thereby inhibiting regeneration from transformed cells. However, with insufficient concentration, the non-transformed cells may overgrow and also inhibit regeneration of transformed cells, allowing the regeneration of escapes (CERVERA *et al.*, 2009; KAPAUN and CHENG, 1999). Thereby, a selective agent must in some way interfere in the normal metabolism of the plant cell but must not affect its subsequent growth and regeneration in whole and fertile plant (QUISEN *et al.*, 2009).

Kanamycin is the most commonly antibiotic used in genetic transformation protocols for *Eucalyptus* species, and it is notable how the sensitivity varies within the species. Most of the works focusing in genetic transformation protocols described 50 mgL^{-1} as the appropriate concentration of Km (AGGARWAL *et al.*, 2011; DIBAX *et al.*, 2010; MATSUNAGA *et al.*, 2012; SILVA *et al.*, 2010). For our clone, this concentration drastically reduced the shoot regeneration, and callus formation was observed on 12.5% of the explants. This result corroborate with Aggarwal *et al.* (2011) which observed that 50 mgL^{-1} of Km resulted in drastic decline in shoot regeneration potential compared to those cultured on Km-free medium on *E. tereticornis* explants. González (2002a), Quisen *et al.* (2009) and Ouyang *et al.* (2012) also observed a decrease on callus formation with the increase of Km concentrations, showing that the gradual increase of Km have reduced cellular division, inhibiting organogenesis and giving the explants a chlorotic or necrotic appearance on *E. camaldulensis*, *E. urophylla*, *E. grandis* and *E . grandis x E . urophylla.*

Indeed, the antibiotic sensitivity varies among the species and type of explant. For *E. globulus* cotyledons and hypocotyls regeneration, 75 mgL^{-1} of Km was indicated to be appropriate (MORALEJO *et al.*, 1998), while 100 $mg.L^{-1}$ allowed an efficient selection for transformed callus of *Eucalyptus globulus* (SERRANO *et al.*, 1996). Contrary, other authors have suggested to use even lower Km concentrations for *Eucalyptus* species as Silva *et al.* (2010), who have suggested levels below 12.5 mgL^{-1} to select *E. saligna* transformed cotyledons and Mullins *et al.* (1997), who suggested 9.0 mg L^{-1} as effective to

select and regenerate *E. camaldulensis.* However, in our work the concentration of 12.5 mgL^{-1} of Km showed to be less toxic to our clone, and we observed 32.8% of explants forming shoots, which can lead to an ineffective selection of the explants on further genetic transformation works.

Generally, aminoglycoside antibiotics are very toxic to plant, animal and fungal cells (MIKI and MCHUGH, 2004; NAP *et al.*, 1992). Kanamycin is a trisaccharide composed of one deoxystreptamine and two glucosamine units (2-deoxystreptamine-6-D-glucosamine), that interacts with the 30S and 50S subunit of ribosomes, inhibiting protein synthesis in eukaryote plastids and mitochondria (MIKI and MCHUGH, 2004; NAP *et al.*, 1992).

Geneticin, a derivate from 3´OH-containing gentamycin, acts on the 80S ribosomal protein to block eukaryotic protein synthesis (YU *et al.*, 2003), similar to Km. But, this last is pointed to be even more toxic for plants than Km, so normally lower concentrations of Gen are used due the high toxicity to plants (GONZÁLEZ, 2002a; KAPAUN and CHENG, 1999).

However, if we compare the Km and Gen experiments, considering the difference of the tested concentrations, we could observed that the callus formation was less affected in the explants cultured on Gen, which allowed more callus and shoot induction than those observed on Km. The toxic effect of 4-8 mgL^{-1} of Gen was higher only than 12.5 mgL^{-1} of Km, showing that for an effective selection of our clone we will need to apply concentrations higher concentrations of Gen than those tested, in order to avoid the regeneration of non-transformed explants during the genetic transformation procedure. In agreement with this observation, González (2002a) reported to have escapes when he used concentrations of 2.5 and 5.0 mgL^{1} of Gen, while 10 mgL^{-1} inhibited shoot formation on genetic transformation of *E. grandis,* and he also described better selection when Km was used.

There are only few reports were Gen was used to select transgenic plants of *Eucalyptus*. When compared Km (100 mgL^{-1}) and Gen (10 mgL^{-1}), Andrade (2001) reported variability on the response among different *Eucalyptus* species, and indicated *E. robusta* and *E. saligna* as the more susceptible to this antibiotic. Geneticin was reported to have high toxicity on Siberian elm *(Ulmus pumila* L.) than other aminoglycoside antibiotics, were 1.0 mgL^{-1} inhibited significantly shoot formation and at 4.0 mg L^{-1} the explants were completely

killed after 1 week (KAPAUN & CHENG, 1999). For *Carica papaya* L. Cv. Tainung No. 2, concentrations higher than 12.5 mgL^{-1} of Gen were necessary to completely inhibit callus growth on non-transformed explants (YU *et al.*, 2003). Moreover, concentrations of 35 and 60 mgL^{-1} of Gen were suitable for callus formation of different sugarcane cultivars (RAZA *et al.*, 2010). When evaluated different Gen concentrations as selective agent for *A. mangium* explants, Xie and Hong (2002) observed delayed of 10 days than the control treatment on callus and shoot proliferation, when cultured on media supplemented with 10-12 mgL^{-1} of Gen, and after 40 days stem segments and shoots cultured with 20 mgL^{-1} or higher turned necrotic.

So according to our results, concentrations above 50 mgL^{-1} of Km had more effect on callus and shoot induction when comparing to the Gen concentrations tested.

4.2. Genetic transformation of clone BRS07-01

Pre-culture and co-culture days did not interfered in the transformation process, at least in the experiments evaluated. However, it should be noticed that in these experiments, we used only 25 Km, what allowed the regeneration of several escapes, and we did not use AS, which later on proved to be essential for the genetic transformation of our clone.

Regarding Km concentration on the transformation protocol, we observed that 50 mgL^{-1} Km was essential to regenerate transgenic plants. Using this concentration we observed 1.3% of TE for leaves explants. This result is in accordance with other reports as mentioned above for other *Eucalyptus* species that pointed this concentration as appropriate for genetic transformation protocols (AGGARWAL *et al.*, 2011; DIBAX *et al.*, 2010; MATSUNAGA *et al.*, 2012; SILVA *et al.*, 2010).

We observed that by adding AS to the genetic transformation process, the TE enhanced, mainly when 50 µM AS was used during the liquid co-culture and 100 µM AS was used on solid co-culture. But, apparently the AS was more effective when at least 100 µM was added to the solid co-culture. Similar to this result, Alcantara *et al.* (2011) reported improvement on *GUS* expression of *E. urograndis* clones when 50 µM of AS was added to the pre and co-culture media. Differently, Spokevicius *et al.* (2005) reported enhancement of GUS

expression adding 100 µM of AS on the bacteria solution before placing with the apical segments of *E. globulus*.

Indeed, phenolic compounds as acetosyringone are indicated to improve plant tissue infection by *Agrobacterium* by inducing the virulence genes in the *vir* region of Ti plasmid (ALCANTARA *et al.,* 2011). Even that plant tissues are natural capable to exudate some *vir*-inducing signals during the *in vitro* culture process due to some stresses, the stimulation by the natural exudates seems not to be sufficient to elicit the *vir* region, turning necessary the addition of exogenous phenolic compounds to the process (ALCANTARA *et al.* 2011).

Beside phenolic compounds, other aspects are highlighted as important to improve TE. Prior to the genetic transformation procedure, small wounds are made on the explants to turn them more vulnerable to the bacteria gateway and infection (KE *et al.,* 2001; MCCULLEN and BINNS, 2006). In response to repair the wounded tissue, the cells of the region show higher cellular division rates and lower pH, and these phenomena have been indicated to increase the TE (MCCULLEN and BINNS, 2006). This observation highlight the importance of pre-culture step before the infection time as well as the effect of co-culture time because it is the substantial moment for the T-DNA transfer and integration on the plant genome (GIRIJASHANKAR, 2011; OUYANG *et al.,* 2012).

Although we could not observe the pre and co-culture days influence, 1 day of pre-culture and 3 days of co-culture may have favored the effectiveness on TE observed on the AS evaluation. Different pre-culture days were reported to improve the *GUS* expression for *Eucalyptus* species as 2 days (AHAD *et al*., 2014; HO *et al*., 1998) and 3 days (ALCANTARA *et al.,* 2011) for *E. camaldulensis* and longer days (4 and 6) for *E. globulus* (MORALEJO *et al.,* 1998). And the importance of co-culture time on genetic transformation was also reported for *E. camaldulensis* (HO *et al.,* 1998) and *E. globulus* (SPOKEVICIUS *et al.,* 2005). In the meantime, after optimizing antibiotic concentrations, pre-culture times (6 days), pH of the inoculation medium (5.6) and co-culture times (6 days), Ouyang *et al.* (2012) reported 7% efficiency in the genetic transformation of *E. urophylla* hypocotyls with *Rs-AFP2* gene. This result is beyond that observed for most of the reported on literature for

Eucalyptus species genetic transformation by *Agrobacterium* and which had passed through indirect organogenesis regeneration.

In other protocols, the authors observed: 1.16-2.33% of transformed shoots of different clones of *E. tereticornins* (AGGARWAL *et al.* 2011), 1.46% for *E. urograndis* (BALIEIRO, 2013), 0.5% for *E. saligna* (DIBAX *et al.,* 2010), an average of 3.98% (MATSUNAGA *et al.,* 2012) and 1.2% (MORALEJO *et al.,* 1998) for *E. globulus.* And even using biobalistic, Sartoretto *et al.* (2002) and Serrano *et al.* (1996) reported difficulties to regenerate transgenic shoots and Balieiro (2013) reported 0.71% of positive transgenes for *E. urograndis.* Most of these works used seeds as source of explants. Higher transformation rates for a clonal material was reported by Chen *et al.* (2001). After they modified and optimized the regeneration protocol they reported transformation rates of 8.1% and 13.5% for sense and antisense quaking aspen *C4H* gene on a clonal material of *E. camaldulensis.*

However, low efficiency is still a main bottleneck for *Eucalyptus* genetic transformation, and the primordial cause is pointed to be the recalcitrance of this genus on *in vitro* procedures (DE LA TORRE *et al.,* 2014; MATSUNAGA *et al.,* 2012; Ribeiro, 2012). The genotype susceptibility to *Agrobacterium* strains and the nature of the explants are pointed to interfere in TE (GONZÁLEZ, 2002a; MACHADO *et al.,* 1997; TOURNIER *et al.,* 2003).

Higher transformation rates was reported using the sonication, where the explants are submitted to sonication while are immersed in an *Agrobacterium* suspension, creating several micro-wounds on the explant and enhancing the bacteria contact among the plant tissue to DNA delivery (SANTARÉM *et al.,* 1998) and infiltration under vacuum to increase the bacteria entrance trough the plant tissue (GONZÁLEZ *et al.,* 2002b).

Combining both methods, sonication and infiltration, Tournier *et al.* (2003) aiming down-regulation of lignin, reached about 10% of integrative and regenerative transformation when submitted leaves explants of *E. urograndis* for 15 s sonication and 5 min of infiltration under vacuum. In this case is also important to consider the fact that they pre-cultured the explants for 2 days and co-cultured for 5 days with 50 µM of AS and added 1 mM of proline at the liquid co-culture medium prior the infection time, which act as an osmoprotectant favoring several virulence genes expression (GALLEGO *et al.,* 2003). González

et al. (2002b) also reported increase of transient *GUS* expression when seed and seedlings of *E. urograndis* where submitted to sonication before and after bacteria inoculation. Silva *et al.* (2011) observed high level of GUS expression on *E. saligna* Sm shoot tip explants after co-culture without or with 100 µM of AS, but these authors did not regenerated transgenic plants. The β-glucoronidase activity and stability was studied by Andrade (2001), he observed that over time the axillary buds submitted to biobalistic showing GUS staining were reduced significantly from 52% to 8.6% after 15 days.

The advantage of using microshoot explants instead of leaves was mentioned by Silva *et al.* (2011) who pointed that they are easier to manipulate due to the size, the high regenerative ability, less occurrence of oxidation, the regeneration phase which is the limiting factor for many elite species and clones (NEHRA *et al.,* 2005) and consequently the reduced chances of somaclonal variation.

Our results confirmed that it is better to use microshoots as explants instead of leaves. By using this type of explant and combining pre-cultured (1 day), 120 seconds of sonication and co-cultured (3 days) with 100 µM AS, the TE was increased up to 20%. This result was much higher than the TE observed from leaves explants. Moreover, the transgenic shoots recovery was much faster, due the unnecessity to wait for all organogenic process.

The sonication-assisted *Agrobacterium*-mediated transformation system (SAAT) has been documented to improve TE. Gallego *et al.* (2003) reported higher *GUS* positives rates (74%) on microshoots of *E. globulus,* using this system and infiltration by vacuum pump and a desiccator. However, they grow the *Agrobacterium* strains with 100 µM of AS and 1 µM of proline. Recently, de la Torre *et al.* (2014) reported 65% *GUS* positive expression of microshoots from a *E. globulus* elite clone also using the combination of SAAT and infiltration. This last two mentioned reports, clearly illustrate that the TE have more probability to increase using microshoots as explants and the SAAT. Although they results were not clear for the exactly overall TE achieved.

5. CONCLUSION

We obtained a highly efficient genetic transformation protocol for clone BRS07-01 of *E. urophylla*. Acetosyringone in the pre or co-culture and initial concentration of 50 mg L^{-1} Kanamycin proved to be essential for genetic transformation. Microshoots were more efficient as source of explants than leaves. In the concentrations we tested, kanamycin was more effective as selective agent than geneticin.

Acknowledgments

The authors wish to thank for the master scholarship sponsored by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and EMBRAPA Forestry (Empresa Brasileira de Pesquisa Agropecuária) for the financially support to this work. And Dr. Estefano Paludzyszyn Filho and Paulo Eduardo Telles dos Santos from Embrapa Forestry fr providing the clone BRS07-01 and technical informationn about it.

6. REFERENCES

AGGARWAL, D.; KUMAR, A.; SUDHAKARA REDDY, M. Agrobacterium tumefaciens mediated genetic transformation of selected elite clone(s) of Eucalyptus tereticornis. **Acta Physiologiae Plantarum**, v. 33, n. 5, p. 1603–1611, 2011. Disponível em: <http://link.springer.com/10.1007/s11738-010-0695-3>. Acesso em: 25/4/2013.

AHAD, A.; MAQBOOL, A.; MALIK, K. OPTIMIZATION OF AGROBACTERIUM TUMEFACIENS MEDIATED TRANSFORMATION IN EUCALYPTUS CAMALDULENSIS. **Pak. J. Bot**, v. 46, n. 2, p. 735–740, 2014. Disponível em: <http://www.pakbs.org/pjbot/PDFs/46(2)/47.pdf>. Acesso em: 10/11/2014.

ALCANTARA, G. B. DE; BESPALHOK FILHO, J. C.; QUOIRIN, M. Organogenesis and transient genetic transformation of the hybrid Eucalyptus grandis × Eucalyptus urophylla Organogênese e transformação genética transiente do híbrido Eucalyptus grandis × Eucalyptus urophylla. **Scientia Agricola**, v. 68, n. 2, p. 246–251, 2011.

ANDRADE, A. D. E. Avaliação de parêmatros que influenciam a transformação genética do Eucalyptus grandis via Agrobacterium. **Tese (Doutorado em Genética e Melhoramento de Plantas**), Escola Superior de Agricultura Luiz de Queirzo - Universidade de São Paulo, p. 82, 2001.

BALIEIRO, F. P. Transformação genética de híbridos de Urograndis. **Dissertação (Mestrado)**, Universidade Federal de Lavras. p. 85, 2013.

CASTELLANOS-HERNÁNDEZ, O. A.; RODRÍGUEZ-SAHAGÚN, A.; ACEVEDO-HERNÁNDZ, G. J.; HERRERA-ESTRELLA, L. R. Genetic Transformation of Forest Trees. In: P. M. Alvarez (Org.); **Genetic Transformation**. p.328, 2011. InTech. Disponível em: <http://www.intechopen.com/books/genetic- transformation/genetic-transformation-of-forest-trees>. .

CERVERA, M.; NAVARRO, L.; PEÑA, L. Gene stacking in 1-year-cycling APETALA1 citrus plants for a rapid evaluation of transgenic traits in reproductive tissues. **Journal of biotechnology**, v. 140, n. 3-4, p. 278–282, 2009.

CHEN, Z.; CHANG, S.; HO, C.; et al. Plant Production of Transgenic Eucalyptus camaldulensis Carrying the Populus tremuloides Cinnamate 4-Hydroxylase Gene C4H. **Taiwan J For Sci**, v. 16, n. 4, p. 249–258, 2001.

CHRIQUI, D.; ADAM, S.; CAISSARD, J.; NOIN, M.; AZIM, A. Shoot regeneration and Agrobacterium-mediated transformation of E. globulus and E. gunni. In: A. Schonau (Org.); IUFRO symposium on intensive forestry: the role of Eucalyptus. Proceedings. **Anais...** . Pretoria, South Africa.p.70–80, 1992.

CTNBIO. Ministério de Ciência, Tecnologia e Inovação. Disponível em: <http://www.ctnbio.gov.br>. .

DIBAX, R.; DESCHAMPS, C.; BESPALHOK FILHO, J. C.; et al. Organogenesis and Agrobacterium tumefacienes-mediated transforation of Eucalypruts saligna with P5CS gene. **Biologia Plantarum**, v. 54, n. 1, p. 6–12, 2010.

FERREIRA, M. E.; GRATTAPAGLIA, D. **Introdução ao uso de marcadores moleculares em análise genética.** 3rd ed. Brasília: EMBRAPA-CERNAGEM Documento 20, 1998.

GALLEGO, P. P.; RODRIGUEZ, R.; DE LA TORRE, F.; VILLAR, B. Genetic Transformation of Eucalyptus globulus. In: S. Espinel; Y. Barredo; E. Ritter (Orgs.); **Sustainable Forestry Wood Products & Biotechnology**. p.163–170, 2003. Vitoria-Gasteiz, Spain: DFA-AFA Press.

GALLEGO, P. P.; RODRIGUEZ, R.; DE LA TORRE, F.; VILLAR, B. Procedimiento para transformar material vegetal procedente de arboles adultos. 2009. Spain.

GHERARDI HEIN, P. R. **G**enetic and environmental control of microfibril angle on eucalyptus wood : its effects on wood traits and implication for selection e Sciences. **Universite Montpellier II - Sciences et Techniques du Languedoc**, 2011.

GIRIJASHANKAR, V. Genetic transformation of eucalyptus. **Physiology and Molecular Biology of Plants**, v. 17, n. 1, p. 9–23, 2011. Disponível em: <http://www.springerlink.com/index/10.1007/s12298-010-0048-0>. Acesso em: 13/3/2013.

GONZÁLEZ, E. R. Transformação genética de *Eucalyptus grandis* e do híbrido *E . grandis x E . urophylla* via *Agrobacterium.* **Tese (Doutorado) Escola Superior de Agricultura "Luis de Queiroz"-** Universidade de São Paulo. p. 93, 2002.

GONZÁLEZ, E. R.; ANDRADE, A. DE; BERTOLO, A. L.; et al. Production of transgenic Eucalyptus grandis × E. urophylla using the sonication-assisted Agrobacterium transformation (SAAT) system. **Functional Plant Biology**, v. 29, n. 1, p. 97– 102, 2002.

GRATTAPAGLIA, D.; VAILLANCOURT, R. E.; SHEPHERD, M.; et al. Progress in Myrtaceae genetics and genomics: Eucalyptus as the pivotal genus. **Tree Genetics and Genomes**, v. 8, n. 3, p. 463–508, 2012.

HO, C.-K.; CHANG, S.-H.; TSAY, J.-Y.; et al. Agrobacterium tumefaciens - mediated transformation of Eucalyptus camaldulensis and production of transgenic plants. **Plant Cell Reports**, v. 17, n. 9, p. 675–680, 1998.

JEFFERSON, R. A. EXPERIMENTAL PROTOCOLS: Assaying Chimeric Genes in Plants: The GUS Gene Fusion System. **Plant Molecular Biology Reporter**, v. 5, n. i, p. 387–405, 1987.

KAPAUN, J. A.; CHENG, Z.-M. Aminoglycoside Antibiotics Inhibit Shoot Regeneration from Siverian Elm Leaf Explants. **HortScience**, v. 34, n. 4, p. 727–729, 1999.

KE, J.; KHAN, R.; JOHNSON, T.; SOMERS, D. A.; DAS, A. High-efficiency gene transfer to recalcitrant plants by Agrobacterium tumefaciens. **Plant Cell Reports**, v. 20, p. 150–156, 2001.

DE LA TORRE, F.; RODRÍGUEZ, R.; JORGE, G.; et al. Genetic transformation of Eucalyptus globulus using the vascular-specific EgCCR as an alternative to the constitutive CaMV35S promoter. **Plant Cell, Tissue and Organ Culture (PCTOC)**, v. 117, n. 1, p. 77–84, 2014.

LLOYD, G.; MCCOWN, B. Commercially-feasible micropropagation of Mountain laurel, Kalmia latifolia, by use of shoot tip culture. **Int. Plant Prop. Soc. Proc.**, v. 30, p. 421–427, 1981.

MACHADO, L. DE O. R.; ANDRADE, G. M.; BARRUETO CID, P. L.; PENCHEL, R. M.; BRASILEIRO, A. C. M. Agrobacterium strain specificity and shoot tumour formation in eucalypt (Eucalyptus grandis x E. urophylla). **Plant Cell Reports**, v.

16, p. 299–303, 1997.

MATSUNAGA, E.; NANTO, K.; OISHI, M.; EBINUMA, H.; et al. Agrobacterium-mediated transformation of Eucalyptus globulus using explants with shoot apex with introduction of bacterial choline oxidase gene to enhance salt tolerance. **Plant cell reports**, v. 31, n. 1, p. 225–35, 2012.

MATSUNAGA, E.; NANTO, K.; OISHI, M.; EBINUMA, H. Agrobacterium-mediated transformation of Eucalyptus globulus using explants with shoot apex with introduction of bacterial choline oxidase gene to enhance salt tolerance. **Plant Cell Reports**, v. 31, p. 225–235, 2012.

MCCULLEN, C. A; BINNS, A. N. Agrobacterium tumefaciens and plant cell interactions and activities required for interkingdom macromolecular transfer. **Annual review of cell and developmental biology**, v. 22, n. May, p. 101–27, 2006.

MIKI, B.; MCHUGH, S. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. **Journal of Biotechnology**, v. 107, p. 193–232, 2004.

MORALEJO, M.; ROCHANGE, F.; BOUDET, A. M.; TEULIÈRS, C. Generation of transgenic Eucalyptus globulus plantlets through Agrobacterium tumefaciens mediated transformation. **Australian Journal of Plant Physiology**, v. 25, n. 2, p. 207–212, 1998.

MULLINS, K. V.; LLEWELLYN, D. J.; HARTNEY, V. J.; STRAUSS, S.; DENNIS, E. S. Regeneration and transformation of Eucalyptus camaldulensis. **Plant Cell Reports**, v. 16, n. 11, p. 787–791, 1997.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Phisiol. Plant**, v. 15, p. 473– 497, 1962.

NAP, J.-P.; BIJVOET, J.; STIEKEMA, W. J. Biosafety of kanamycin-resistant transgenic plants. **Transgenic Research**, v. 1, n. 6, p. 239–249, 1992.

NEHRA, N. S.; BECWAR, M. R.; ROTTMANN, W. H.; et al. Forest biotechnology: Innovative methods, emerging opportunities. **In Vitro Cellular & Developmental Biology - Plant**, v. 41, n. 6, p. 701–717, 2005.

OUYANG, L.; HE, W. H.; HUANG, Z. C.; et al. Introduction of the Rs-AFP2 gene into Eucalyptus urophylla for resistance to Phytophthora capsici. **Journal of Tropical Forest Science**, v. 24, n. 2, p. 198–208, 2012.

PENNA, S.; SÁGI, L.; SWENNEN, R. Positive selectable marker genes for routine plant transformation. **In Vitro Cell. Dev. Biol**, v. 38, p. 125–128, 2002.

QUISEN, R.; OLIVEIRA, Y. DE; PILEGGI, M.; CUQUEL, F. Selective Agent and A . tumefaciens Overgrowth-control Antibiotics in Eucalyptus camaldulensis

Cotiledonary Culture. **Braz. Arch. Biol. Technol.**, v. 52, n. 6, p. 1485–1492, 2009.

RAZA, G.; ALI, K.; MUKHTAR, Z.; et al. The response of sugarcane (Saccharum officinarum L) genotypes to callus induction, regeneration and different concentration of the selective agent (geneticin-418). **African Journal of Biotechnology**, v. 9, n. 51, p. 8739–8747, 2010.

RIBEIRO, S. R. Regeneração e transformação em Eucalyptus grandis Regeneração e transformação em Eucalyptus grandis. **Dissertação (Mestrado), Universidade de Lisboa**, 2012.

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS, T.; SPRING, H. L. C. **Molecular Cloning: A Laboratory Manual.** 2nd ed. ed. Colc Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press, 1989.

SANTARÉM, E. R.; TRICK, H. N.; ESSIG, J. S.; FINER, J. J. Sonication-assisted Agrobacterium- mediated transformation of soybean immature cotyledons: optimization of transient expression. **Plant Cell Reports**, v. 17, n. 10, p. 752–759, 1998.

SARTORETTO, L. M.; CID, L. P. B.; BRASILEIRO, A. C. M. Biolistic transformation of Eucalyptus grandis x E. urophylla callus. **Functional Plant Biology**, v. 29, n. 8, p. 917 – 924, 2002.

SERRANO, L.; ROCHANGE, F.; SEMBLAT, J. P.; et al. Genetic transformation of Eucalyptus globulus through biolistics : complementary development of procedures for organogenesis from zygotic embryos and stable transformation of corresponding proliferating tissue. **Journal of experimental botany**, v. 47, n. 295, p. 285–290, 1996.

SILVA, A. L. L. DA; OLIVEIRA, Y. DE; COSTA, J. DA L.; et al. Shoot tip and cotyledon explants of Eucalyptus saligna Sm. cultivated on different kanamycin levels. **Journal of Biotechnology and Biodiversity**, v. 1, n. 1, p. 1–5, 2010.

SILVA, A. L. L.; OLIVEIRA, Y. DE; COSTA, J. DA L.; et al. Preliminary results for genetic transformation of shoot tip of *Eucalyptus saligna* Sm . via Agrobacterium tumefaciens. **Journal of Biotechnology and Biodiversity**, v. 2, n. February, p. 1–6, 2011.

SPOKEVICIUS, A. V.; VAN BEVEREN, K.; LEITCH, M. A.; BOSSINGER, G. Agrobacterium-mediated in vitro transformation of wood- producing stem segments in eucalypts. **Plant Cell Reports**, v. 23, p. 617– 624, 2005.

TOURNIER, V.; GRAT, S.; MARQUE, C.; et al. An efficient procedure to stably introduce genes into an economically important pulp tree (Eucalyptus grandis × Eucalyptus urophylla). **Transdenic Research**, v. 12, p. 403–411, 2003.

WALTER, C.; MENZIES, M. Genetically modified trees. In: **Forest and Genetically Modified Trees**, ed. FAO, Rome, p. 3-18. 2010.

XIE, D. Y.; HONG, Y. Agrobacterium-mediated genetic transformation of Acacia mangium. **Plant Cell Rep**, v. 20, p. 917–922, 2002.

YADAV, S. K.; KATIKALA, S.; YELLISETTY, V.; et al. Optimization of Agrobacterium mediated genetic transformation of cotyledonary node explants of Vigna radiata. **SpringerPlus**, v. 1, n. 59, p. 8, 2012.

YU, T.-A.; YEH, S.-D.; YANG, J.-S. Comparison of the effect of Knamaycin and geneticin on regeneration of papaya from root tissue. **Plant Cell, Tissue and Organ Culture**, v. 74, p. 169–178, 2003.

CHAPTER III- Can endophytic bacteria cause constraints in genetic transformation of *Eucalyptus* via *Agrobacterium tumefaciens*?

ABSTRACT

Eucalyptus species are known to be very recalcitrant to *Agrobacterium tumefaciens* genetic transformation. Since we observed endophytic bacteri growing in *Eucalyptus* spp. micropropagated plantlets, the objective of this work was to evaluate if these microorganisms can be somehow involved in the low efficiency of the genetic transformation of *Eucalyptus urophylla via Agrobacterium tumefaciens*, either by producing antagonistic substances, or by physical barrier to the *Agrobacterium* strain. We isolated endophytic bacteria from the clone BRS07-01 of *E. urophylla* maintained *in vitro* for over 1,5 year. After sequencing we could identify two of the isolates as *Stenotrophomonas maltophilia*. Histochemical *GUS* assay of this isolate revealed that this isolate produces betaglucuronidase, suggesting that this test could be leading to false positives in the genetic transformation of *Eucalyptus* using the *uidA* as a marker gene. We analyzed the isolate for the production of substances or characteristics that could be inhibiting the *A. tumefaciens* infection or growing. We observed that the isolate GB701 produces biofilm that could be a barrier to the *Agrobacterium* infection. Furthermore, although we could not observe inhibition by the paring test, the *Stenotrophomonas* genus is known to produce several antibiotic compounds, responsible for the biological control of soil microorganisms, and we suggest that they could be somehow interfering in the *A. tumefaciens* infection.

Key words: *Stenotrophomonas;* biofilm; IAA production;

1. INTRODUCTION

Many microorganisms taxa have the ability to live as plant endophytes, such as Rhizobia which is probably the most studied plant-bacteria interaction bacteria (TAGHAVI *et al.,* 2009). Endophytes can have beneficial, detrimental and/or neutral interaction with the plants (HUNG and ANNAPURNA, 2004). They live in symplast or apoplast in the plants (GUNSON and SPENCER-PHILLIPS, 1994; HAKIMANA *et al.,* 2011), receiving nutrients from the host

plant and mutually conferring direct or indirectly support and/or increasing the plant health and growth, without substantively harming it (DAWWAM *et al.,* 2013; HADDAD *et al.,* 2013; HAKIZIMANA *et al.,* 2011).

The benefits of endophytes for plant growth and health varies from production of plant growth regulators (PGRs), promotion of mechanisms that involves nitrogen fixation, syntheses of particular compounds to facilitate the uptake of certain nutrients as mineral phosphates, overcoming phytotoxic effects caused by environmental contamination, suppression of stress ethylene synthesis by 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, induction of resistance to phytopathogens through competition for space and nutrients, antibiosis, production of siderophores, hydrolytic enzymes, inhibition of pathogen-produced enzymes or toxins, and through systemic induction of plant defense mechanisms (AHMAD *et al.,* 2014; HUNG and ANNAPURNA, 2004; DAWWAM *et al.,* 2013; ZHUANG *et al.,* 2007; VAN DER LELIE *et al.,* 2009; TAGHAVI *et al.,* 2009). Even so, the plant-bacteria symbiosis association is socioeconomically beneficial, since they improve the plant overall performance, enhancing multiple applications as biomass production, carbon sequestration and phytoremediation (TAGHAVI *et al.,* 2009).

However*,* in micropropagation, sometimes the presence of endophytes can be harmful for in vitro culture of the plantlets. In vitro conditions create a perfect environment that facilitates the growth of endophytes, primarily because of the complex media which contain several nutrients (ASIF *et al.,* 2013; JENA and SAMAL, 2011). This can be a problem when the growth of endophytes overgrow the plant tissues, competing for the medium nutrients leading to the plant death or even disturb the conduct of the experiments (ASIF *et al.,* 2013; ULRICH *et al.,* 2008; CAREY *et al.,* 2015).

The presence of endophytic microorganisms can be even more harmful if these can somehow hinder the genetic transformation via *Agrobacterium tumefaciens.* Once these microorganisms are responsible for pathogen tolerance in plants, they could be involved in defense mechanisms against *A. tumefaciens* virulence or infection process.

This work aimed to isolate and characterize endophytic bacteria from clone BRS07-01 of *Eucalyptus urophylla* and to investigate their influence on genetic transformation of this clones via *Agrobacterium tumefaciens*.

2. MATERIAL AND METHODS

2.1. Plant material

In vitro micropropagated plantlets of clone BRS07-01 of *Eucalyptus urophylla* were used as explants for endophytic bacteria isolation. The clone was maintained for 1.5 year on MS medium (MURASHIGE and SKOOG, 1962) containing 30 g L^{-1} sucrose, 0.88 µM BAP, 0.2 mg L^{-1} myo-inositol and 7 gL^{-1} of agar, in a growth room at 23±2°C under white fluorescent light with an irradiance of 40 $\mu mol.m^{-2}.s^{-1}$ and 16 hours of photoperiod at Embrapa Forestry, Colombo- Brazil.

2.2. Isolation of endophytic bacteria from the clone BRS07-01 of E. urophylla

To isolate endophytic bacteria, leaves with petiole of the plantlets, without visual presence of contamination, were placed on solid lysogeny broth (LB) (SAMBROOK *et al.,* 1989) medium (containing 10 g L^{-1} of NaCl, 10 g L^{-1} tryptone and 5 g L^{-1} yeast extract, pH 7,0) and incubated in the dark at 23°C to allow bacteria growth. After 3 days, the bacteria growing around the explants were transferred to a fresh solid LB medium and cultivated for another 3 days at the same conditions, to purify the colonies. Then, 5 single colonies were picked and maintained at 4°C on solid LB medium for further tests.

2.3. Effect of temperature on growth of isolate GB701

We evaluated the effect of three different temperatures on the growth rate of isolate GB701: 23, 25 and 28 ºC. The isolates were streaked on solid LB medium on agar plates and cultivated for 3 days in the dark, to observe the bacteria growth. This experiment consisted on three replications.

2.4. Sequencing of the isolate GB701

The phylogenetic assignment of one isolate (GB701) was carried out by sequence analysis of the 16S rRNA gene. Total genomic DNA extraction was done as described by Vicente *et al*. (2008), using silica:celite (2:1) for

maceration in CTAB (Cetyltrimethylammonium bromide) and CIA (chloroform:isoamyl alchol). After DNA precipitation, the DNA was quantified using NanoDrop 1000 (Spectrophotometer Thermo Scientific) and by agarose gel (1%) quantification. For the sequence analysis of the 16S rRNA gene, universal primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') positions 8 to 27 and rD1 (5'-AAGGAGGTGATCCAGCC-3'), positions 1541 to 1525 Y1 (5'-TGGCTCAGAACGAACGCTGGCGGC-3') and rD1 (5'-GCTTAAGGAGGTGATCCAGCC-3') from *E. coli* strain K12 described by Weisburg *et al.* (1991) were used to amplify (amplicons with 1500 bp). The PCR reactions were performed under the following conditions: PCR mixture (25 µl) containing 1.0 U Taq DNA polymerase, 2.0 mM $MgCl_2$, 1x PCR buffer, 2.0mM of each dNTP, 0.4 mM of each forward and reverse primer (Invitrogen, Brazil) and approximately 50 ng template DNA and submitted to 1 cycle of 94ºC for 2 min; 35 cycles of 94ºC for 1 min, 58ºC for 1 min and 72ºC for 2 min, 1 cycle of 72ºC for 6 min. The resulting PCR products were ligated into pGEM- T Easy vectors (Promega, France) and cloned using *E.coli* DH5α (Invitrogen™, Brazil). Then the plasmids containing the 16S rDNA inserts were purified using the Wizard miniprep DNA purification system and submitted to another PCR reaction (Promega, USA). The amplicons integrity was observed on 1% agarose gel electrophoresis, photographed by a Cannon PowerShot S2 IS camera under Syngene Bio Imaging program. After that for the amplicons purification before sequencing, they were submitted to PEG purification: 50 µL of 20% PEG were pipetted on the PCR tubes containing the amplicons, gently mixed by pipetting up & down and rested for 30 min at 37ºC. After that they were centrifuged for 20 min at 13000 rpm and the supernatant were discarded. One hundred and twenty five microliters (125 µL) of freezing ethanol 80% was added and centrifuged for 2 min more and discarded the supernatant. This step was repeated with freezing ethanol 96% and then the pellet was left to dry at 45ºC for 1 h. After all, the pellet was suspended with 15 µL of water milliQ and left at room temperature for 2 h. The sequencing of the amplicons was carried out using the same primers in ABI3130 sequencer from Biochemistry department of UFPR.

Table1. Reference strains from *Stenotrophomonas* gender.

	Name	Reference Number	Source of substrate/ Geografic Origen	Genbank
1	*Stenotrophomonas acidaminiphila*	ATCC 700916	upflow anaerobic sludge blanket (UASB) reactor	AF273080.1
2	*Stenotrophomonas africae*	ATCC 700475	-	U62646.1
3	*Stenotrophomonas chelatiphaga*	CCUG 57178	-	EU573216.1
4	*Stenotrophomonas daejeonensis*	JCM 16244	South Korea	GQ241320.1
5	*Stenotrophomonas dokdonensis*	CIP 108839	Dokdo, a Korean island	DQ178977.1
6	*Stenotrophomonas ginsengisoli*	KCTC 12539	isolated from a ginseng field	DQ109037.1
7	*Stenotrophomonas humi*	DSM 18929	Soil	AM403587.1
8	*Stenotrophomonas koreensis*	JCM 13256	isolated from compost, South Korea	AB166885.1
9	*Stenotrophomonas maltophilia*	ATCC 13637	oropharyngeal region of patient with cancer	AB294553.1
10	*Stenotrophomonas nitritireducens*	A. TCC BAA-12	from biofilters	AJ012229.1
11	*Stenotrophomonas pavanii*	CBMAI 564	Brazilian sugar cane varieties	FJ748683.2
12	*Stenotrophomonas rhizophila*	ATCC BAA-473	-	AJ293463.1
13	*Stenotrophomonas terrae*	DSM 18941	soil	AM403589.2

(-) no observed. Source: http://www.bacterio.net/stenotrophomonas.html

Alignment and Phylogenetic construction

The obtained sequences edition was performed using the Staden program version 1.6 (BONFIELD *et al.*, 2002). The sequences were compared with sequences from isolates deposited in the reference database (NCBI (National Center for Biotechnology Information -. Http://www.ncbi.nlm.nih.gov/) and by BLAST program (ALTSCHUL *et al.*, 1997) to detect similarity between the sequences. To alignment, the Mafft program (http://mafft.cbrc.jp/alignment/server/) was used, performing a visual inspection

by MEGA software version 5.1 (TAMURA *et al.*, 2011). To phylogenetic analysis all the 12 known strains of *Stenotrophomonas* gender, using *E.coli* O157:H7 (STEC O157:H7) as outgroup based on Kaparullina *et al.* (2009) (Table 1). The Maximum Likelihood phylogenetic tree was built with 1000 bootstrap, using the evolutionary model Kimura 2 Parameters using the MEGA program to version 5.1 for final edition (TAMURA *et al*., 2011).

2.5. Electronic microscopy for detection of endogenous bacteria

For electronic microscopy detection leaves with petiole explants and callus formed from leaves of clone BRS07-01 were fixed according to (BOMBLIES *et al.,* 2008). The explants were immersed in FAA fixative (3.7% v/v formaldehyde, 50% ethanol, 5% acetic acid) and left overnight (~18h) at 4ºC. Afterwards, explants were rinsed 3 times in 25 mM sodium phosphate buffer (pH 7) before dehydrating in an ethanol series (30%, 50%, 70%, 95% and 100% dry, 30 min each step). The 100% dry ethanol was repeated twice and the explants were stored overnight at 4°C. After that, the explants were dried using the critical point technique and coated with gold. The photomicrograph was taken by a JEOL (JSM 6360-LV) scanning electron microscope (JEOL Ltd., Japan) at the Electronic Microscopy Centre, UFPR.

2.6. Histochemical Gus assay

The histochemical assays were carried out in *in vitro* plantlets of clone BRS07-01 under micropropagation, on calli of the same clone and in the isolate GB701 cultured on petri dishes.

For this test, isolate strains were streaked on a petri dish with LB medium containing 250 mg L^{-1} of X-gluc compound (inoculated after autoclavation) and cultivated at the same conditions for 48 h. After that we evaluate qualitatively the *GUS* staining. To evaluate the endogenous *GUS* staining for the clone BRS07-01 we performed histochemical analyses as described by Jefferson (1987). *In vitro* shoot explants and calli explants from *E. urophylla* were immersed on a solution containing 10mM Na2EDTA.H2O, 0,1%

Triton X-100, 0,1 M NaH2PO4, 0,5 M K3Fe(CN)6, and 250 µg.mL-1 5-bromo-4-chloro-3-indolyl β-D-glucuronic acid (X-GLUC, Gold Biotecnhology Inc, USA) and incubated for 16 h at 37°C. After that, the explants were washed several times with ethanol 70% to remove chlorophyll, and the *GUS* staining was observed and photographed.

To promote callogenesis of clone BRS07-01 of *Eucalyptus urophylla*, leaves were collected from *in vitro* grown plants and inoculated in petri dishes with 20 mL of the callus induction medium (CIM) [WPM (LLOYD and MCCOWN, 1981) containing 30 gL^{-1} sucrose, 0.5 µM TDZ, 0.1 µM NAA, 0,1 mg L^{-1} myo-inositol, 500 mg L^{-1} PVP and 7 gL^{-1} agar] for 30 days at 23±2°C in dark. The pH of all media was adjusted to 5.8 before autoclaving for 20 min at 120°C 1atm.

2.7. Antagonist activity of isolate GB701 over Agrobacterium tumefaciens strain EHA105

Different methods were used to observe antagonistic activity of the isolate GB701 against *A. tumefaciens* strain EHA105.

Experiment I: First a dual culture assay was evaluated. *Agrobacterium tumefaciens* was streaked in solid LB medium and disks of the isolate, previously grown on solid LB medium, were placed on the center of the petri dish with *A. tumefaciens,* and cultured for 48 h at 28°C.

Experiment II: To observe antagonist activity, both bacteria strains were streaked side by side in solid LB medium and cultured at the same conditions.

Experiment III: To observe if any compound produced by the isolate had any interference on the *A. tumefaciens* growth, we cultured the isolate overnight with 100 mg L^{-1} of tryptophan. The log phase bacteria solution was centrifuged for 10 min at 5985 g to obtain a cell-free culture filtrate. Then the filtrate was added to solid LB medium (v/v 20%) and the *Agrobacterium* strain was streaked over the medium and cultured for 48h at the same conditions, to observe the growth. A control plate was done with LB medium with no filtrate and a LB medium with the filtrate without *Agrobacterium* inoculation. All the evaluations were done in triplicate.

2.8. Antibiotic sensitivity test of isolate GB701

We tested the sensitivity of the isolate GB701 to five different antibiotics, all of them in 3 different concentrations (except for gentamycin): amoxicillin (150, 180 and 200 mg L^{-1}), cefotaxime (180, 200 and 300 mg L^{-1}), kanamycin (12.5, 25 and 50 mg L^{-1}) and gentamycin (5 mg L^{-1}). The antibiotics solutions were introduced after autoclavation on solid LB medium. The strain was inoculated on the medium and incubated for 72 h at 23°C and then evaluate the sensitivity. The evaluation measure was based on the bacteria growth, which the ability to grow in the medium was considered no sensitivity to the antibiotic. To the evaluation we performed a control treatment without antibiotics, and the experiment was conducted in triplicate for each isolate.

2.9. Biofilm formation

Formation of biofilm was tested as described by Barbosa *et al.* (2005) with some modifications. The test was carried out in 15 ml polypropylene tubes containing 2-3 ml of LB medium and 100 µl of an exponential-phase culture grown overnight of the isolate GB701 in the same medium. The tubes were incubated for 24 h without agitation at 23°C. The cultures were discarded and the tubes were rinsed with distilled water and stained with a solution of 1% crystal violet for 15 min and subsequently washed to remove excess stain. The isolate test was in duplicates and the controls consisted of an uninoculated tube and a tube inoculated with exponential-phase cells for 10 min before washing. The tubes were evaluated for the presence of a purple ring at the tube wall.

2.10. Indole acetic acid (IAA) production

The ability of the isolate GB701 to produce IAA was determined qualitatively and quantitatively. The isolate was cultured overnight on LB medium at 23°C, and them 200 µL of this bacterial culture were inoculated on a 50 mL Erlenmeyer flask containing 25 ml of LB medium with tryptophan (0.1 %), and incubated for 4 days at 23ºC at 80 rpm, in dark. After that, the cultures were centrifuged at 958 g for 30 min, and 2 ml of the supernatant was mixed with 2

drops of orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 1 mL 0.5M FeCl3) was added, and the mixture was allowed to stand for 25 min. The indole acetic acid (IAA) production was measured qualitatively, by observation of the production of rose color, and quantitatively by the measure of the intensity of the rose color at 530 nm using a spectrophotometer (UV Espectrophotometer 1800, Shimadzu). For quantitative determination, a standard curve (from 0-100 µg/L) was also developed with a standard solution of pure indole-3-acetic acid. The experiment was conducted in triplicate.

3. RESULTS

3.1. Isolation and sequencing of the isolate

After three days culture on LB medium, we could observe the overgrowth o endophytic bacteria around the leaves of *E. urophylla* clone BRS07-01. The bacteria were streaked on new LB plates and we could observe the formation of several single colonies. From those, we purified 5 isolates, which were used for further PCR amplification.

The DNA was isolated and the 16S region was amplified for sequencing. We were able to sequence two isolates and both were identified as *Stenotrophomonas africae* (Figure 1). This species was first identified by Drancourt *et al*. (1997), but in 2004, Coenye and collegues compared this strain type with *S. maltophilia* and with other species from this genus, and concluded that *S. africae* is a later synonym of *S. maltophilia.* Therefore, our isolates were a *Stenotrophomonas maltophilia*. Yet we chose one of them, GB701, for the further analyses.

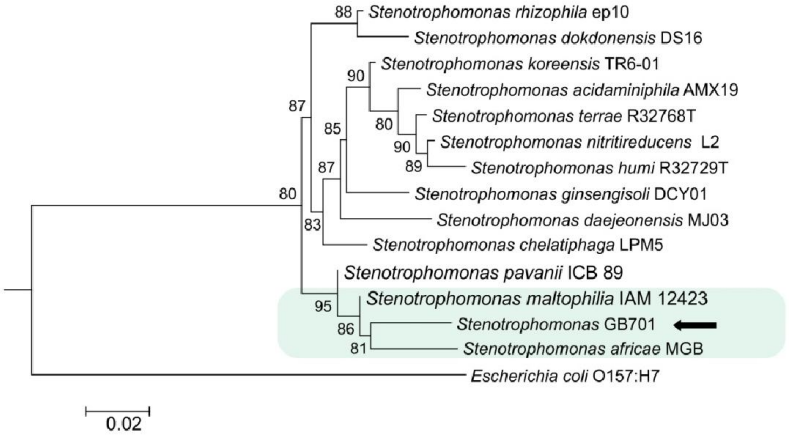

Figure1. 16S rDNA-based dendrogram showing the phylogenetic position of *Stenotrophomonas africae* (isolate GB701) among other *Stenotrophomonas* species and *E. coli*.

3.2. Effect of temperature on the isolate GB701 growth

We tested the growth of isolate GB701 under three different temperatures. We could not observe differences in the bacteria growth among the temperatures, suggesting that the isolate presents plasticity and can growth at least in temperatures ranging from 23 to 28°C.

3.3. Histochemical Gus Assay of shoots and callus from clone BRS07-01 of Eucalyptus urophylla and of the isolate GB701 of Stenotrophomonas maltophilia

The isolate GB701 was able to metabolize the X-gluc substrate, what could be observed by the blue staining of colonies growing on LB medium after 48 h of culture (Fig 2 A). Blue staining was also observed in internal tissues of shoots of micropropagated plantlets of clone BRS07-01, especially at the vascular tissue, and on the callus forming from its leaves (Fig. 2 B and C).

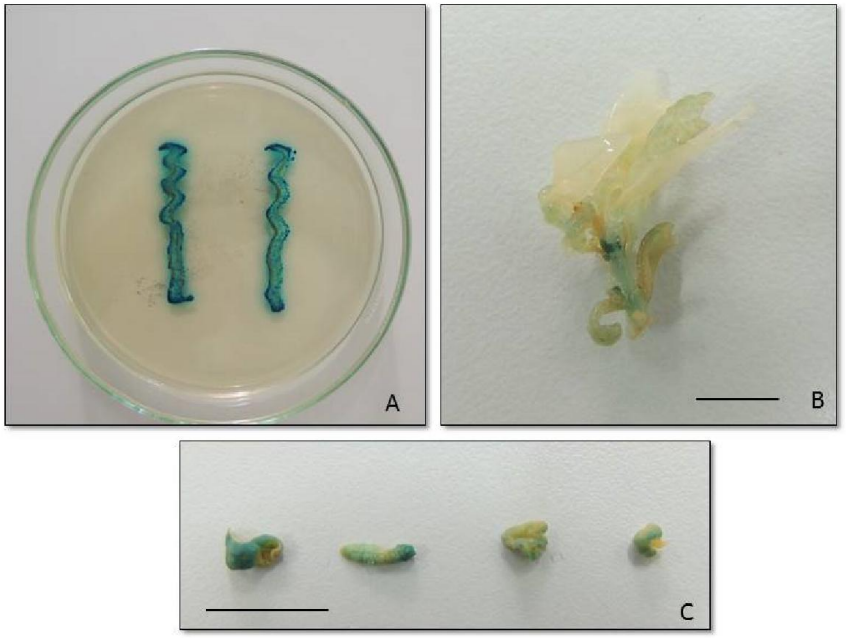

Figure 2. (A) Positive endogenous *GUS* for GB701. (B) Positive endogenous *GUS* for formed callus of leaves explants from BRS07-01 clone (Bar=0.5 cm); (C) Positive endogenous for shoot explants BRS07-01 clone (Bar= 1.0 cm).

3.4. *Antagonist activity from the isolate GB701of Stenotrophomonas maltophilia over Agrobacterium tumefaciens*

No antagonistic effect was observed between the isolate GB701 and *A. tumefaciens* EHA105. Both bacteria grow normally, without any apparent interference with the dual culture and even by culturing side by side (Fig. 3 A and B). However, even that the *A. tumefaciens* could grow in LB containing the cell-free filtrate, we observed that the bacteria pattern was quite different over the *Agrobacterium* control plate (Fig. 3C), showing that the cell-free filtrate could have a substance that probably interfere on *Agrobacterium* growth.

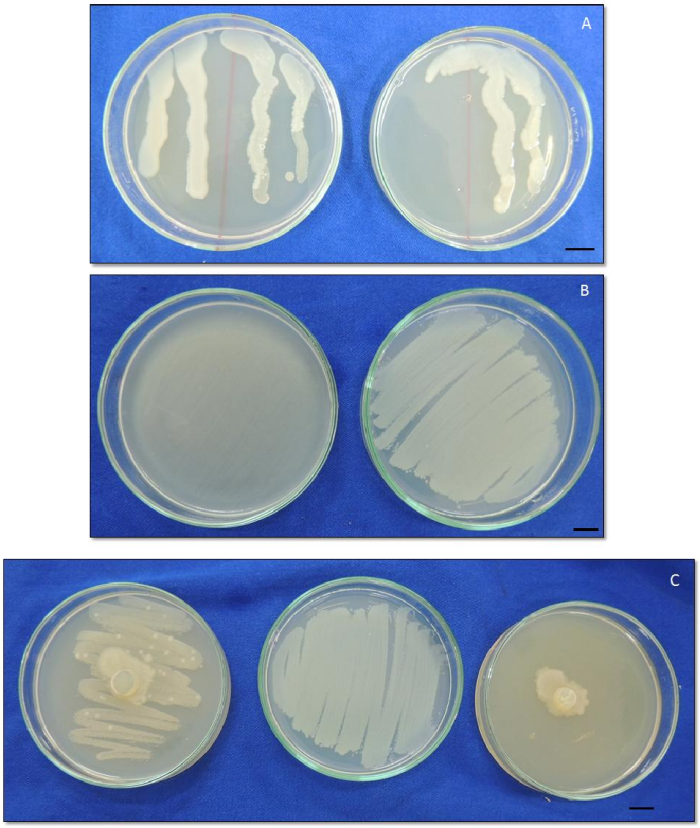

Figure 3. Antagonist activity from the isolate GB701 of *Stenotrophomonas maltophilia* over *Agrobacterium tumefaciens.* (A): first plate with GB701 strain (on left) and *A. tumefaciens* (on right), the second plate is the control with only *A. tumefaciens.* (B) first plate *A. tumefaciens* growing on LB medium with 20% of GB701 supernatant and the second plate is the *A. tumefaciens* control plate, growing on LB medium. (C) Pareament test from the isolate GB701 over *A. tumefaciens,* the first plate is showing the growth of the isolate disc over the *Agrobacterium* strain, the next represents the *A. tumefaciens* control plate (on midle) and the isolate GB701 control growth. Bar= 1.0 cm.

3.5. Antibiotic resistance test of isolate GB701

The isolate GB701 showed different sensitivity levels for the antibiotics tested, although not among the concentrations of each one (Table 2). On media containing kanamycin and geneticin, the isolate could growth as well as in the control plates without antibiotics. On media containing amoxicillin and cefotaxime the bacteria could grow at the concentrations evaluated.

Table 2. Sensitivity of the isolate GB701 over four different antibiotics at different concentrations.

Antibiotic	Concentration (mg L^{-1})	Sensitivity
Amoxicillin	150	+
	180	+
	200	+
Cefotaxime	180	+
	200	+
	300	+
Kanamycin	25	-
	50	-
Geneticin	5	-

(+) shows sensitivity to the antibiotic which the isolate couldn't grow and (-) shows that the isolate could grow in the antibiotic and demonstrated no sensitivity.

3.6. Electronic microscopy image and Biofilm formation

The MVE image showed the presence of bacteria micro-colonies at high density between the intercellular space of the leaves and callus tissues (Fig. 4). The bacteria could be classified as having bacillus morphology, characteristic of *Stenotrophomonas maltophilia.* Colonies of bacteria forming a biofilm matrix could be observed on leaves and callus.

The isolate GB701 was tested for biofilm production at polypropylene tube walls. We observed a stained purple ring at the tube wall, confirming the formation of a biofilm (Fig. 5).

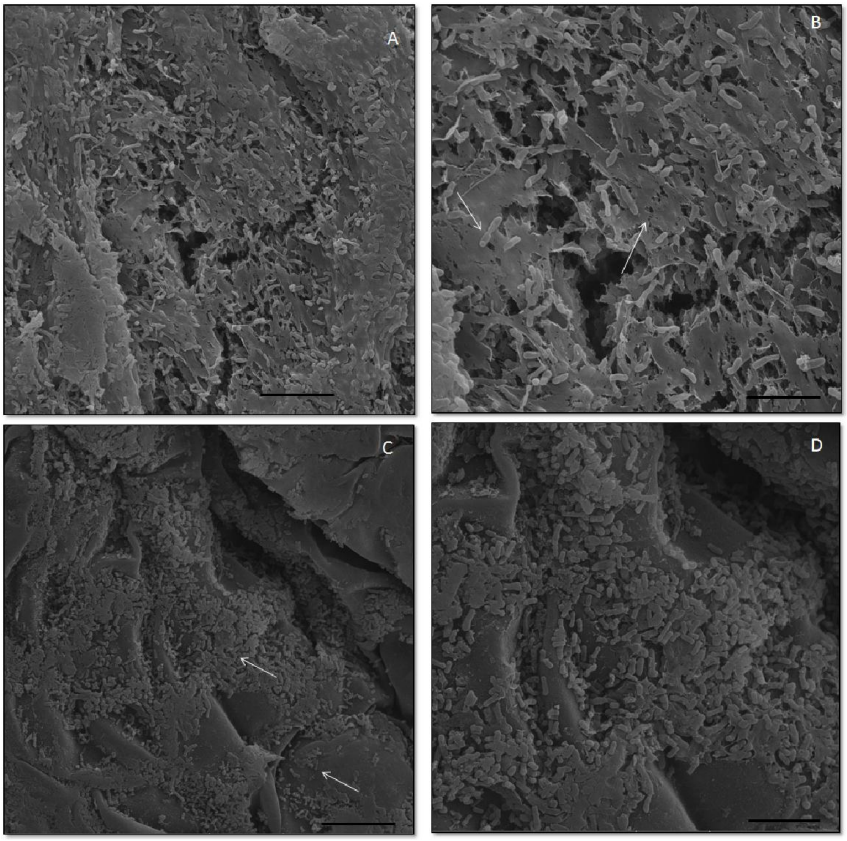

Figure 4. MVE images showing the presence of bacteria colonization in the interface of leaf tissue of the *E. uropylla* BRS07-01 clone. (A and B) images from leaf of *in vitro* micropropagated shoots of clone BRS07-01, arrows showing bacteria with bacillus shape and presence of a biofilm matrix. Bars = 10 and 5 µm, respectively. (C and D) images from callus of leaves from clone BR07-01, arrows showing presence of bacteria colonies. Bars = 10 and 5 µm, respectively.

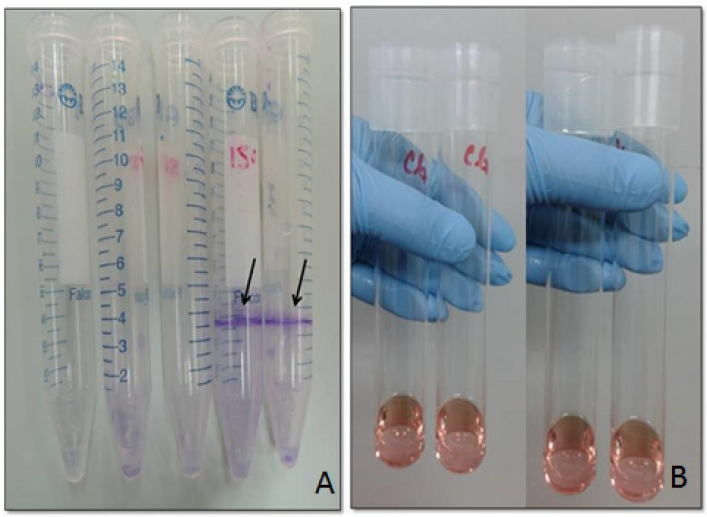

Figure 5. (A) Confirmation of biofilm formation by the isolates (tube 1- negative control, tube 2 and 3- control with isolate incubated 10 min before staining, tube 4 and 5- confirmation of biofilm formation by the pellet formed (arrows) from GB701 isolate. (B) Qualitative result confirmation of IAA production from both isolates.

3.7. IAA production by isolate GB701

We observed the production of 0.055 µg mL^{-1} of the auxin IAA after 4 days of culture on LB medium containing 25 mL of LB medium with tryptophan (0.1%), and measured using Salkowski's reagent at 530 nm.

4. DISCUSSION

Endophytic microorganisms live in symbiosis with plants in nature, and can help plants in their plasticity, to survive biotic and abiotic stresses. In tissue culture, these organisms live inside the culture, probably with the same benefits as in the nature. However, under certain circumstances, they can be harmful and interfere on the *in vitro* plants growth (LUNA *et al.,* 2013).

In our laboratory, we have *E. urophylla* clones under micropropagation for over 1.5 year. Although we cannot observe endophytic bacteria growing in the cultures, we observed the presence of bacteria in the medium after some time in organogenesis experiments from leaves. As the aim of our organogenesis was to use the explants for genetic transformation, and our

efficiency via *Agrobacterium tumefaciens* transformation was low (2%), we decided to investigate if the endophytic bacteria of our clone could somehow hinder the growth or the infection process of the *A. tumefaciens.*

After placing leaves of the *E. urophylla* clone BRS07-01 on LB plates, we isolated 5 colonies, grown for further studies. By sequencing of 16s rRNA region, one of our isolates, GB701 showed 100% homology to *Stenotrophomonas africae* (synonym of *S. maltophilia)*, a gram negative bacteria. This genus has at least known 12 species, and the most reported species is the *S. maltophilia* (RYAN *et al.,* 2009). This bacteria is found throughout the environment and seems to be well adapted to live in association with both plant and human hosts, been also identified as an endophytic bacteria (TAGHAVI *et al.,* 2009; RYAN *et al.,* 2009; POMPILIO *et al.,* 2010). Bacteria of this genus have benefic effects for plant growth and health, the breakdown of natural and manmade pollutants that are central to bioremediation and phytoremediation strategies and the production of biomolecules of economic value, as well as detrimental effects, such as multidrug resistance, in human pathogenic strains (RYAN *et al.,* 2009). *Stenotrophomonas* spp. was also found in poplar tissue culture in association with other bacteria genus, as *Paenibacillus* and *Methylobacterium* (ULRICH *et al.,* 2008) and in *Ilex paraguariensis* shoot cultures (LUNA *et al.*, 2013). The symbiosis interaction with plant may suggest that this genus can confer plant tolerance over pathogens.

Once our isolate GB701 was identified, we tested the influence of temperature on its grow. We tested 23, 25 and 28°C, once these are the temperatures used for the micropropagation of *E. urophylla* clone BRS07-01 and growth of *A. tumefaciens,* respectivelly. We did not observe difference on the growth on the three temperatures, suggesting that this factor will not influence the growth of our isolate during the genetic transformation process of *A. tumefaciens*.

Once we previously observed that after genetic transformation of *E. urophylla* clone BRS07-01 the explants stained blue in histochemical test even in the control plants, we decided to test our isolate GB701 for the expression of betaglucuronidase gene, as well as the micropropagated plantlets of clone BRS07-01 and calli induced on its leaves.

The *uidA* gene is most widely used reporter gene in transgenic plant research (XIONG *et al.,* 2011) owing to be simple, easy to quantify, highly sensitive assay and cost-effective (GILISSEN *et al.,* 1998; MIKI and MCHUGH, 2004; XIONG *et al.,* 2011). On the other hand, was expected little or no detectable of beta-glucuronidase in higher plants (JEFFERSON, 1987; SUDAN *et al.,* 2006). However, there are reports of endogenous *GUS*-like activity in vegetative tissues (SOLÍS-RAMOS *et al.,* 2010), which can be a problem on plant genetic transformation because can lead to "false positive" results (FIOR and GEROLA, 2009; HU *et al.,*1990; SOLÍS-RAMOS *et al.,* 2010; SUDAN *et al.,* 2006; TOR, 1992; WILSON *et al.,* 1992).

Therefore, some authors attributed the endogenous *GUS* background activity in plants assays to endophytic microorganisms or microbial contamination during the enzyme assay (RAINERI *et al.,* 1990; WILSON *et al.,* 1992). Other two researchers also attributed to endophytes the *GUS* background, Wilson *et al.* (1992) and Tor (1992). This last author found that the endophytic bacteria from aseptically micropropagated shoot cultures of the monocotyledonous genus *Dioscorea* (yam), which later were identified as two species of *Curtobacterium*, could express *GUS* with a variety of glucuronide substrates, and was responsible for the false positive results during plant transformation experiments. Meanwhile some *Stenotrophomonas* species were reported to be positive for β-Glucosidase activity among the *S. maltophilia* (KAPARULLINA *et al*., 2009), *S. rhizophila* (WOLF *et al*., 2002), *S. terrae* (HEYLEN *et al*., 2007), *S. acidaminiphila* (ASSIH et al., 2002), which supports our histoquemical *GUS* assay result, pointing that our isolate has β-Glucosidase activity and may be responsible for the positive *GUS* response in eucalyptus tissues.

In the in vitro plant, we observed that the blue stain was concentrated mostly in the vascular tissue (Fig. 2B) suggesting that the concentration of the endophytic bacteria is higher in these tissues. The presence of endophytic bacteria was also confirmed on leaves of clone BRS07-01 after blue staining and on the callus forming from leaves on organogenesis experiment.

The MVE images showed high concentrations of endophytic bacteria within the plant tissues, allowing the visualization of the bacteria biofilm (Fig. 4). As defined by Costerton *et al.* (1995) "biofilms are a matrix-enclosed bacterial

populations adherent to each other and/or to surfaces or interfaces". This is one of the mechanisms from bacteria phenotypic plasticity to respond through different environmental stimuli to favor their growth (COSTERTON *et al.,* 1995). Bounding near the surface it suggests a strong survival and/or selective advantage for the bacteria community than living at their free-ranging counterparts. Moreover, in an aqueous environment the nutrients tend to concentrate near to a solid surface (DUNNE Jr, 2002).

The biofilm functions as a cooperative consortium, in a relatively complex and coordinated manner, which requires collective bacterial behavior (DAVEY and O'TOOLE, 2000) and confer protection to the bacteria communities from the adverse environmental conditions and from biological and chemical threats antibacterial agents (COSTERTON *et al.,* 1995; SILVA *et al.,* 2011). Basically the biofilm is composed by microbial cells and the EPS (extracellular polymeric substance), which can be up to 90% of the composition (DONLAN, 2002), and protein, nucleic acids, and other substances (DAVEY and O'TOOLE, 2000). Even so, this matrix creates a three-dimensional force field that surrounds, anchors, and protects surface-bound bacteria (DUNNE Jr, 2002), and acts as an ion exchanger preventing the physically access of certain antimicrobial agents into the biofilm surround area (DAVEY and O'TOOLE, 2000; GILBERT *et al.,* 1997). This EPS mechanism may explain the recalcitrance to genetic transformation of this *E. urophylla* clones. The biofilm could be acting as a layer above the plant cell tissues, merely preventing the *Agrobacterium* approach and infection by providing a physical barrier, blocking the access to the gene transfer to the plant genome.

Another species of *Stenotrophomonas* has already been reported as capable of producing biofilms. Pompilio *et al.* (2010) cultured on epithelial cells (IB3-1 cells) and also on polystyrene surfaces several strains of this genus and observed biofilm formation. After 24 h of infection, they also observed by scanning electron microscopy and confocal laser scanning micrographs, that *S. maltophilia* microcolonies were scattered across almost all IB3-1 cells and over the extracellular matrix.

The dual culture evaluation that we used did not revealed any antagonisms between the two bacteria. But when cultured on LB medium containing the cell-free filtrate, the *A. tumefaciens* growth showed a different

pattern from the control, suggesting that it is possible that our isolate produces a compound which can interfere on the *Agrobacterium* growth, as diffusible and volatile compounds as reported by Chaurasia *et al*. (2005) which observed that diffusible and volatile compounds of *Bacillus subtilis* strain caused antagonist activity over fungus causing morphological abnormalities in fungal structures.

According to Chaurasia *et al.* (2005), bacteria antagonism activity can be confered by a variety of compounds of microbial origin (bacteriocins, enzymes, toxic substances, volatiles, and others). The *Stenotrophomonas* genus is reported to produce bacteriocin compounds (LIIU *et al.,* 2013). Liu *et al.* (2013) discovered a novel phage tail-like bacteriocin, named maltocin P28, produced by *S. maltophilia* strain P28*.* Using one of the open reading frames genes of the maltocin P28, Dong *et al.* (2015) produced a recombinant endolysin (P28), which contain the lysozyme-like superfamily conserved, and tested the antibacterial spectrum against various Gram-negative and Gram-positive bacteria. After further analysis they observed that this endolysin was able to lyse all of the tested Gram-positive bacteria and against three Gram-negative bacteria (from a totally of 5), showing to have a hydrolysis activity against peptidoglycan. As it known, endolysins acts by digesting the peptidoglycan from the bacterium cell wall, causing a hole and consequently a lethal effect (DONG *et al.,* 2015; FISCHETTI, 2010). So, the authors believe that endolysin P28 was not effective for all the Gram-negative bacteria, once on these bacteria present an outer membrane, which is impermeable to macromolecules and makes a physical barrier for endolysin to access the peptidoglycan layer (DONG *et al.,* 2015).

Once we normally use antibiotics in two different phases of genetic transformation of Eucalyptus, as a selective agent to regenerate events, and to kill the *Agrobacterium tumefaciens*, we tested the most used antibiotics to evaluate their effect on *Stenotrophomonas maltophilia* growth and if this growing could be somehow explaining the mechanisms of interference among *S. maltohilia* and *A. tumefaciens* during genetic transformation of *E. urophylla.* We evaluated the antibiotic sensitivity from isolate GB701 based on the concentrations used in genetic transformation protocols. The isolate showed the same pattern of sensitivity/resistance as *A. tumefaciens*, suggesting that this is probably not the main factor of interference. The isolate was sensitive for all the

tested concentrations of amoxicillin and cefotaxime, which are used to control and avoid the *Agrobacterium* growth after the co-culture step (ASIF *et al.,* 2013; GREWAL *et al*., 2006; YU and WEI, 2008). This two antibiotics have a wide spectrum against gram-negative bacteria (ASIF *et al.,* 2013) and acts by inhibiting bacterial cell wall synthesis and rising cell lysis (LEIFERT *et al.,* 1990). Contrariwise, they were not sensitive for kanamycin and geneticin, which are mostly used in plant genetic transformation as selective antibiotics, to kill the non-transformants explants. However, the major problem is that we are not dealing only with free living organisms but also with biofilms bacteria communities, which turn them more resistant to antimicrobial agents, and requires higher concentrations of antibiotics, over three or four orders higher, to achieve bactericidal activity against adherent organisms (DUNNE Jr, 2002; GILBERT *et al.,* 1997).

Although we could not observe an antagonistic response from *S. maltophilia* against *A. tumefaciens,* the use of endophytic microorganisms as biological control for pathogen is becoming more regularly (HAKIZIMANA *et al.,* 2011). The genus *Stenotrophomonas* its well-known to be an excellent natural antagonist to pathogen infection, growth and survival (MESSIHA *et al.,* 2007). Jakobi *et al.* (1996) reported an antifungal macrocyclic lactam antibiotic production called maltophilin (β-lactamase L1) from *S. maltophilia* R551-3 which confer resistance, antagonistic mechanism and inhibit the growth of various saprophytic, zoopathogenic and phytopathogenic fungi.

In 2009, Van der Lelie and collegues, showed that this same strain was capable to produce a large spectrum of syderophors related to iron uptake, including syderophors receptors for ferricalcaligin and ferrichrome, produced by several phytopathogenic fungi (ARDON *et al.,* 1997). This characteristic turns out the capability to compete with pathogenic microorganisms for ferrous iron, which is often a major microbial growth limiting factor, and control their growth (VAN DER LELIE *et al.,* 2009). Still, the *S. maltophilia* R551-3 strain was also observed to grow on media with mannitol as sole carbon source (TAGHAVI *et al*., 2009), which can lead to a possible defense ability to the host plant against mannitol-secreting pathogenic fungi by metabolizing mannitol (VAN DER LELIE *et al.* (2009).

Also, plant associated bacteria, as *Stenotrophomonas* genre, are related to have biocontrol effects against other soilborne microorganisms pathogens by space competition, production of inhibitory allelochemicals and induction of systemic resistance in host plants (HRYNKIEWICZ and BAUM, 2012; COMPANT *et al.,* 2010).

Several endophytic bacteria are able to synthetize plant growth regulators, such as IAA. Plant growth and development is strictly related to plant growth regulators, which acts as chemical messengers (MARTINEZ-VIVEROS *et al.,* 2010). For their use, plant synthesize these PGRs themselves, but still awarded to count with those synthesized by the inhabitants of rhizosphere or even from endophytic bacteria which have the ability to produce some PGRs, as the auxin IAA (ALI *et al.,* 2010). For this reason, we investigated if our isolate GB701is able to produce IAA.

Generally, IAA synthesis occurs via three alternative pathways using tryptophan as the precursor (TAGHAVI *et al.,* 2009), and several bacteria genus have been shown to produce IAA in presence of L-tryptophan (ALI *et al.,* 2010; DAWWAM *et al.,* 2013). In this study, we confirm the IAA production by tryptophan as precursor from our isolate at the concentration of 0.05 µg ml^{-1}. As well, many reports mentioned IAA production by different bacteria species (ALI *et al.,* 2010; EGAMBERDIEVA, 2008; MATIRU *et al.,* 2013). From seventy three bacteria isolates from rice root, Matiru *et al.* (2013) identified ten isolates capable to produce IAA. Ali *et al.* (2010) showed IAA production in presence of L-trytophan from a range of 1.16-8.22 µg ml^{-1}, from different non-symbiotic plant growth promoting rhizobacteria genera. Afterwards, these same bacteria showed to increase IAA levels when inoculated with *Vigna radiate* (L.) seedlings in presence of tryptophan, but this increase might have impact negatively, because no concurrent increase in shoot length and root growth was observed. Taghavi *et al.* (2009) described IAA production from different endophytic bacteria from poplar trees under greenhouse conditions and observed that some strains as *S. proteamaculans* 568, *P. putida* W619, and *Enterobacter* sp. strain 638 induced rapidly and more denser roots when inoculated with the plants. Actually, auxin sensitivity varies from plant to plant and tissues, and there is a tiny line from positive to negative impacts through high concentrations of IAA (SPAEPEN *et al.,* 2007).

Auxin concentration on the medium as well as produced by the plant or endophytes has been shown to influence on *A. tumefaciens* infection process. Earlier studies where pre-treatment of explants with either auxin alone or both auxin and cytokinin increase T-DNA transfer efficiency and stable transformation (LEE *et al.,* 2009; KRENS *et al.,* 1996; CHATEAU *et al.,* 2000). It is believed that the increase in susceptibility is due to induced cell division caused by the phytohormones, leading to physiological state modification that could influence *Agrobacterium* attachment and stable transformation, by increasing the T-DNA integration (GOHLKE and DEEKEN, 2014).

It has been shown that indole-3-acetic acid (IAA) has an impact on agrobacterial virulence by inhibiting vir gene induction and growth of agrobacteria on high concentrations of the auxin (25–250 μM) (LIU and NESTER, 2006). Application of 1 μM IAA, a concentration found in wounded and uninfected *Arabidopsis* stems, stimulated growth of *Agrobacterium* cells, whereas growth stimulation vanished at 10 μM and higher IAA concentrations (personal communication, J. Ludwig-Mueller, Technical University Dresden, Germany cited by GOHLKE and DEEKEN, 2014). Although our bacteria produced IAA at low concentrations, it is to be noticed that the medium contained auxin and the explants their selves were probably also synthetizing this PGR.

We could characterize the isolate and suggest that it is probably influencing the genetic transformation of our clone BRS07-01. However, one question still needs to be answered. Is it possible to micropropagate *Eucalyptus* without endophytic bacteria, or reducing severely their concentration in order to undermine their effect on *A. tumefaciens* growth or virulence without leading the plant to death because of its dependence of these bacteria?

5. CONCLUSION

We isolated and characterized an endophytic bacteria from *Eucalyptus urophylla* clone BRS07-01 clone and identified it as *Stenotrophomonas maltophilia*. The isolate is able to produce biofilm, which can be acting as a physical barrier to *Agrobacterium tumefaciens* transformation of our clone. Although we did not observe antagonistic effect on pareament tests, this genus

is known to produce antagonistic compounds against microorganisms and could be somehow acting against the virulence of *A. tumefaciens.* The isolate also showed to expressed betaglucuronidase gene, interfering in the histochemical assays in transgenic plants of this clone. From the results we suggest that this isolate has a negative effect on the genetic transformation of clone BR07-01 by rather its physical, chemical or biological characteristics.

Acknowlegemnt

We would like to thank Dra. Vânia Vicente to provide the materials and equipment for the sequence analysis. And also, would like to acknowledge CAPES for the Master scholarship (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior) and EMBRAPA Forestry (Empresa Brasileira de Pesquisa Agropecuária) for the financial support.

6. REFERENCES

AHMAD, Z.; LONE, R.; SHUAB, R.; KOUL, K. K. Techneques for the Isolation and In-vitro Screening of Plant Growth Promoting Rhizobacteria (PGPR) from Rhizosphere. **Middle-East Journal of Scientific Research**, v. 22, n. 1, p. 72–77, 2014.

ALI, B.; SABRI, A. N.; HASNAIN, S. Rhizobacterial potential to alter auxin content and growth of Vigna radiata (L.). **World Journal of Microbiology and Biotechnology**, v. 26, n. 8, p. 1379–1384, 2010.

ALTSCHUL, S. F.; MADDEN, T. L.; SCHAFFER, A. A.; et al. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. **Nucleic Acids Research, Oxford,** v. 25, p. 3389– 402, 1997.

ARDON, O.; WEIZMAN, H.; LIBMAN, J.; et al. Iron uptake in Ustilago maydis: Studies with fluorescent ferrichrome analogues. **Microbiology-Uk**, v. 143, p. 3625–3631, 1997.

ASIF, M.; EUDES, F.; RANDHAWA, H.; et al. Cefotaxime prevents microbial contamination and improves microspore embryogenesis in wheat and triticale. **Plant Cell Rep**, v. 31, p. 1637–1646, 2013.

ASSIH, E. A., OUATTARA, A. S., THIERRY, S., CAYOL, J.-L., LABAT, M. & MACARIE, H. Stenotrophomonas acidaminiphila sp. nov., a strictly aerobic bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. **Int J Syst Evol Microbiol**, v. 52, p. 559–568, 2002.

BARBOSA, T. M.; SERRA, C. R.; LA RAGIONE, ROBERTO M WOODWARD, M. J.; HENRIQUES, A. O. Screening for Bacillus Isolates in the Broiler Gastrointestinal Tract. **Applied and environmental microbiology**, v. 71, n. 2, p. 968–978, 2005.

BOMBLIES, K.; SHUKLA, V.; GRAJAM, C. Scanning Electron Microscopy (SEM) of plant tissues. **Cold Spring Harb Protoc**, v. 3, n. 4, p. 1–3, 2008.

BONFIELD, J.; BEAL, K.; JORDAN, M.; CHEN, Y.; STADEN, R. **The Staden Package Manual.** Copyright, 2002.

CAREY, S. B.; PAYTON, A. C.; MCDANIEL, S. F. A Method for Eliminating Bacterial Contamination from in Vitro Moss Cultures A METHOD FOR ELIMINATING BACTERIAL. **Applications in Plant Science**, v. 3, n. 1, p. 1–5, 2015.

CHATEAU, S.; SANGWAN, R. S.; SANGWAN-NORREEL, B. S. Competence of Arabidopsis thaliana genotypes and mutants for Agrobacterium tumefaciensmediated gene transfer: role of phytohormones. **J. Exp. Bot.**, v. 51, p. 1961–1968, 2000.

CHAURASIA, B.; PANDEY, A.; PALNI, L. M. S.; et al. Diffusible and volatile compounds produced by an antagonistic Bacillus subtilis strain cause structural deformations in pathogenic fungi in vitro. **Microbiological Research**, v. 160, n. 1, p. 75–81, 2005.

COENYE, T.; VANLAERE, E.; FALSEN, E.; VANDAMME, P. Stenotrophomonas africana Drancourt et al. 1997 is a later synonym of *Stenotrophomonas maltophilia* (Hugh 1981) Palleroni and Bradbury 1993. **Int. J. Syst. Evol. Microbiol.**, v.a 54, p. 1235–1237, 2004.

COMPANT, S.; CLÉMENT, C.; SESSITSCH, A. Plant growth-promoting bacteria in the rhizo- and endosphere of plants: Their role, colonization, mechanisms involved and prospects for utilization. **Soil Biology and Biochemistry**, v. 42, n. 5, p. 669–678, 2010.

COSTERTON, J. W.; LEWANDOWSKI, Z.; CALDWELL, D. E.; KORBER, D. R.; LAPPIN-SCOTT, H. M. Microbial biofilms. **Annual review of microbiology**, v. 49, p. 711–45, 1995. Disponível em: <http://www.annualreviews.org/doi/pdf/10.1146/annurev.mi.49.100195.003431\nhttp://www.ncbi.nlm.nih.gov/pubmed/8561477>. .

DAVEY, M. E.; O'TOOLE, G. A. Microbial biofilms: from ecology to molecular genetics. **Microbiology and molecular biology reviews**, v. 64, n. 4, p. 847–67, 2000. Disponível em: <http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=99016&tool=pmcentrez&rendertype=abstract>. .

DAWWAM, G. E.; ELBELTAGY, A.; EMARA, H. M.; ABBAS, I. H.; HASSAN, M. M. Beneficial effect of plant growth promoting bacteria isolated from the roots of potato plant. **Annals of Agricultural Sciences**, v. 58, n. 2, p. 195–201, 2013. Faculty of Agriculture, Ain Shams University. Disponível em: <http://dx.doi.org/10.1016/j.aoas.2013.07.007>. .

DONG, H.; ZHU, C.; CHEN, J.; et al. Antibacterial activity of *Stenotrophomonas maltophilia* endolysin P28 against both Gram-positive and Gram-negative bacteria. **Frontiers in Microbiology**, v. 6, n. 1299, 2015.

DONLAN, R. M. Biofilms: Microbial life on surfaces. **Emerging Infectious Diseases**, v. 8, n. 9, p. 881–890, 2002.

DRANCOURT, M.; BOLLET, C.; RAOULT, D. Stenotrophomonas africana sp. nov., an Opportunistic Human Pathogen in Africa. **Int J Syst Evol Microbiol**, v. 47, p. 160–163, 1997.

DUNNE JR, W. M. Bacterial Adhesion: Seen Any Good Bio lms Lately? **Clinical Microbiology Reviews**, v. 15, n. 2, p. 155–166, 2002.

EGAMBERDIEVA, D. Plant Growth Promoting Properties of Rhizobacteria Isolated from Wheat and Pea Grown in Loamy Sand Soil. **Turk J Biol**, v. 32, p. 9–15, 2008.

FIOR, S.; GEROLA, P. D. Impact of ubiquitous inhibitors on the *GUS* gene reporter system: evidence from the model plants Arabidopsis, tobacco and rice and correction methods for quantitative assays of transgenic and endogenous *GUS*. **Plant methods**, v. 5, p. 19, 2009.

FISCHETTI, V. A. Bacteriophage endolysins: a novel anti-infective to control 368 Gram-positive pathogens. **Int. J. Med. Microbiol.**, v. 300, p. 357–362, 2010.

GILBERT, P.; DAS, J.; FOLEY, I. Biofilms susceptibility to antimicrobials. **Adv. Dent. Res.**, v. 11, p. 160–167, 1997.

GILISSEN, L.; METZ, P.; STIEKEMA, W.; NAP, J. Biosafety of E. coli beta- glucuronidase (*GUS*) in plants. **Transgenic Res**, v. 7, p. 157–163, 1998.

GOHLKE, J.; DEEKEN, R. Plant responses to Agrobacterium tumefaciens and crown gall development. Front. **Front. Plant Sci.**, v. 5, n. 155, p. 11, 2014.

GUNSON, H. E.; SPENCER-PHILLIPS, P. T. N. Latent bacterial infections: epiphytes and endophytes as contaminants of micro propagated plants. In: J. R. Nicholas (Org.); **Physiology, growt and development of plants in culture.** p.379–396, 1994.

HADDAD, N.; KRIMI, Z.; RAIO, A. Endophytic bacteria from weeds promote growth of tomato plants in vitro and in greenhouse . **Endophytes in Biotechnology and Agriculture**, p. 1, 2013.

HAKIZIMANA, J. D.; GRYZENHOUT, M.; COUTINHO, T. A.; VAN DEN BERG, N. Endophytic diversity in Persea americana (avocado) trees and their ability to display biocontrol activity against Phytophthora cinnamom. **Proceedings VII World Avocado Congress 2011**, p. 1–10, 2011.

HEYLEN, Kim; VANPARYS, Bram; PEIRSEGAELE, Filip; LEBBE, Liesbeth and VOS, Paul De. Stenotrophomonas terrae sp. nov. and Stenotrophomonas humi sp. nov., two nitrate-reducing bacteria isolated from soil Kim. **International Journal of Systematic and Evolutionary Microbiology**, v. 57, p. 2056–2061, 2007.

HRYNKIEWICZ, K.; BAUM, C. The Potential of Rhizosphere Microorganisms to Promote the Plant Growth in Disturbed Soils. In: A. Malik; E. Grohmann (Orgs.); **Environmental Protection Strategies for Sustainable Development**. Hardcover. p.606, 2012.

HU, C. Y.; CHEE, P. P.; CHESNEY, R. H.; et al. Intrinsic *GUS*-like activities in seed plants. **Plant cell reports**, v. 9, n. 1, p. 1–5, 1990.

HUNG, P. Q.; ANNAPURNA, K. ISOLATION AND CHARACTERIZATION OF ENDOPHYTIC BACTERIA IN SOYBEAN (GLYCINE SP .). , v. 101, p. 92–101, 2004.

JAKOBI, M.; WINKELMANN, G.; KAISER, D.; et al. Maltophilin: a new antifungal compound produced by S*tenotrophomonas maltophilia* R3089. **The Journal of antibiotics**, v. 49, n. 11, p. 1101–4, 1996.

JEFFERSON, R. A. EXPERIMENTAL PROTOCOLS: Assaying Chimeric Genes in Plants: The *GUS* Gene Fusion System. **Plant Molecular Biology Reporter**, v. 5, n. i, p. 387–405, 1987.

JENA, R. C.; SAMAL, K. C. Endogenous microbial contamination during In vitro culture of sweet potato [Ipomoea batatas (L .) Lam]: identification and prevention. , v. 7, n. 6, p. 1725–1731, 2011.

KAPARULLINA, E.; DORONINA, N.; CHISTYAKOVA, T.; TROTSENKO, Y. Stenotrophomonas chelatiphaga sp. nov., a new aerobic EDTA-degrading bacterium. **Systematic and Applied Microbiology**, v. 32, n. 3, p. 157–162, 2009.

KRENS, F. A.; TRIFONOVA, A.; PAUL KEIZER, L. C.; HALL, R. D. The effect of exogenously-applied phytohormones on gene transfer efficiency in sugarbeet (Beta vulgaris L.). **Plant Sci.**, v. 116, p. 97–106, 1996.

LEE, C.-W.; EFETOVA, M.; ENGELMANN, J. C.; et al. Agrobacterium tumefaciens promotes tumor induction by modulating pathogen defense in Arabidopsis thaliana. **The Plant cell**, v. 21, n. 9, p. 2948–62, 2009.

LEIFERT, C.; WAITES, W.; NICHOLAS, J.; KEETLEY, J. Yeast contaminants of micro propagated plant cultures. **J Appl Bacteriol**, v. 69, p. 471–476, 1990.

LIU, J.; CHEN, P.; ZHENG, C.; HUANG, Y.-P. Characterization of Maltocin P28, a Novel Phage Tail-Like Bacteriocin from *Stenotrophomonas maltophilia*. **Applied and Environmental Microbiology**, v. 79, n. 18, p. 5593–5600, 2013.

LIU, P.; NESTER, E. W. Indoleacetic acid, a product of transferred DNA, inhibits vir gene expression and growth of Agrobacterium tumefaciens C58. **Proc. Natl. Acad. Sci. U.S.A.**, v. 103, p. 4658–4662, 2006.

LLOYD, G.; MCCOWN, B. Commercially-feasible micropropagation of Mountain laurel, Kalmia latifolia, by use of shoot tip culture. **Int. Plant Prop. Soc. Proc.**, v. 30, p. 421–427, 1981.

LUNA, C.; ACEVEDO, R.; COLLAVINO, M.; et al. Endophytic bacteria from Ilex paraguariensis shoot cultures : localization , characterization , and response to isothiazolone biocides. **In Vitro Cell Dev. Biol.**, v. 49, p. 326–332, 2013.

MARTINEZ-VIVEROS, O.; JORQUERA, M. A.; CROWLEY, D. E.; GAJARDO, G.; MORA, M. L. Mechanisms and practical considerations involved in plant growth promotion by rhizobacteria. **Soil Sci. Plant Nutr.**, v. 10, n. 3, p. 293–319, 2010.

MATIRU, V. N.; NG, J.; NYAMBATI, V. C. S. Isolation and

Characterisation of Bacterial Root Endophytes with Potential to Enhance Plant Growth from Kenyan Basmati Rice. **American International Journal of Contemporary Research**, v. 3, n. 4, p. 25–40, 2013.

MESSIHA, N. A. S.; VAN DIEPENINGEN, A. D.; FARAG, N. S.; et al. *Stenotrophomonas maltophilia*: a new potential biocontrol agent of Ralstonia solanacearum, causal agent of potato brown rot. **European Journal of Plant Pathology**, v. 118, n. 3, p. 211–225, 2007.

MIKI, B.; MCHUGH, S. Selectable marker genes in transgenic plants: applications, alternatives and biosafety. **Journal of Biotechnology**, v. 107, p. 193–232, 2004.

MURASHIGE, T.; SKOOG, F. A revised medium for rapid growth and bioassays with tobacco tissue cultures. **Phisiol. Plant**, v. 15, p. 473– 497, 1962.

POMPILIO, A.; CROCETTA, V.; CONFALONE, P.; et al. Adhesion to and biofilm formation on IB3-1 bronchial cells by *Stenotrophomonas maltophilia* isolates from cystic fibrosis patients. **BMC microbiology**, v. 10, p. 102, 2010.

RAINERI, D.; BOTTINO, P.; GORDON, M.; NESTER, E. Agrobacterium-mediated transformation of Oryza sativa (Oriza sativa L.). **Biotechnology**, v. 8, p. 33–38, 1990.

RYAN, R. P.; MONCHY, S.; CARDINALE, M.; et al. The versatility and adaptation of bacteria from the genus Stenotrophomonas. **Nature reviews. Microbiology**, v. 7, n. 7, p. 514–525, 2009. Nature Publishing Group.

SAMBROOK, J.; FRITSCH, E. F.; MANIATIS, T.; SPRING, H. L. C. **Molecular Cloning: A Laboratory Manual.** 2nd ed. ed. Colc Spring Harbor, N. Y.: Cold Spring Harbor Laboratory Press, 1989.

SILVA, M. S.; DE SOUZA, A. A; TAKITA, M. A; LABATE, C. A; MACHADO, M. A. Analysis of the biofilm proteome of Xylella fastidiosa. **Proteome Science**, v. 9, n. 1, p. 58, 2011.

SOLÍS-RAMOS, L. Y.; GONZÁLEZ-ESTRADA, T.; ANDRADE-TORRES, A.; GODOY-HERNÁNDEZ, G.; CASTAÑO DE LA SERNA, E. Endogenous *GUS*-like activity in Capsicum chinense Jacq. **Electronic Journal of Biotechnology**, v. 13, n. 4, 2010.

SPAEPEN, S.; VANDERLEYDEN, J.; REMANS, R. Indole-3-acetic acid in microbial and microorganism-plant signaling. FEMS Microbiol Rev 31:425–448. **FEMS Microbiol Rev**, v. 31, p. 425–448, 2007.

SUDAN, C.; PRAKASH, S.; BHOMKAR, P.; JAIN, S.; BHALLA-SARIN, N. Ubiquitous presence of beta-glucuronidase (*GUS*) in plants and its regulation in some model plants. **Planta**, v. 224, n. 4, p. 853–64, 2006.

TAGHAVI, S.; GARAFOLA, C.; MONCHY, S.; NEWMAN, L.; HOFFMAN, A.; et al. Genome Survey and Characterization of Endophytic Bacteria Exhibiting a Beneficial Effect on Growth and Development of Poplar Trees. **Applied and Environmental Microbiology**, v. 75, n. 3, p. 748–757, 2009.

TAGHAVI, S.; GARAFOLA, C.; MONCHY, S.; NEWMAN, L.; HOFFMAN, A.; et al. Genome survey and characterization of endophytic

bacteria exhibiting a beneficial effect on growth and development of poplar. **Appl. Environ. Microbiol.**, v. 75, p. 748–757, 2009.

TAMURA, K.; PETERSON, D.; PETERSON, N.; et al. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. **Molecular Biology and Evolution**, v. 28, n. 10, p. 2731–9, 2011.

TOR, M. Endophytic bacteria expressing beta-glucuronidase cause false positives in transformtion of Dioscorea species. **Plant Cell Rep**, v. 11, p. 452–456, 1992.

ULRICH, K.; STAUBER, T.; EWALD, D. Paenibacillus—a predominant endophytic bacterium colonising tissue cultures of woody plants. **Plant Cell, Tissue and Organ Culture**, v. 93, n. 3, p. 347–351, 2008.

ULRICH, K.; ULRICH, A.; DIETRICH, E. Diversity of endophytic bacterial communities in poplar grown under field conditions. **FEMS Microbiol Ecol**, v. 63, p. 169–180, 2008.

VAN DER LELIE, D.; TAGHAVI, S.; MONCHY, S.; et al. Poplar and its Bacterial Endophytes: Coexistence and Harmony. **Critical Reviews in Plant Sciences**, v. 28, n. 5, p. 346–358, 2009.

VICENTE, V. A.; ATTILI-ANGELIS, D.; PIE, M. R.; et al. Environmental isolation of black yeast-like fungi involved in human infection. **Studies in Mycology**, p. 137–144, 2008.

WEISBURG, W. G.; BARNS, S. M.; PELLETIER, D. A.; LANE, AND D. J. 16S ribosomal DNA amplification for phylogenetic study. **J. Bacteriol.**, v. 173, p. 697– 703, 1991.

WILSON, K.; JEFFERSON, R.; HUGHES, S. The Escherichia coli *gus* operon: Induction and expression of the *gus* operon in Escheri- chia coli and the occurrence and use of *GUS* in other bacteria. In: S. Gallagher (Org.); ***GUS* Protocls: Using the *GUS* gene as report**. New York, NY: Academic Press I. p.7– 40, 1992. nc.

WOLF, A., FRITZE, A., HAGEMANN, M. & BERG, G. Stenotrophomonas rhizophila sp. nov., a novel plant-associated bacterium with antifungal properties. **Int J Syst Evol Microbiol**., v. 52, p.1937–1944, 2002.

XIONG, A.-S.; PENG, R.-H.; ZHUANG, J.; et al. A thermostable β-glucuronidase obtained by directed evolution as a reporter gene in transgenic plants. **PloS one**, v. 6, n. 11, p. e26773, 2011.

YU, Y.; WEI, Z. Influences of cefotaxime and carbenicillin on plant regeneration from wheat mature embryos. **Biol Plant**, v. 52, p. 553–556, 2008.

ZHUANG, X.; CHEN, J.; SHIM, H.; BAI, Z. New advances in plant growth-promoting rhizobacteria for bioremediation. , v. 33, p. 406–413, 2007.

ANEX 1

Table. 1. Deviations values of statistical test and significance levels from the experiment evaluating the effect of Kanamycin as selective agents on indirectly organogenesis of clone BRS07-01, after 30 days.

Variation Sources	Calllus formation	Soots formation	Oxidation frequency	Antocyanin frequency
Trat	D=61,19 <0,001***	D=97,23 <0,001***	D=23,79 <0,001***	D=149,14 <0,001***

[ns] descriptive no significant level of 5% probability; ** Significant at 1% probability; *** Significant at 0.1% probability;

Table. 2. Deviations values of statistical test and significance levels from the Experiment evaluating the effect of Kanamycin as selective agents on indirectly organogenesis of clone BRS07-01, after 90 days.

Variation Sources	Calllus formation	Soots formation	Oxidation frequency	Antocyanin frequency
Trat	D=17,157 <0,001***	D=4,805 0,028*	D=20,98 <0,001***	D=87,508 <0,001***

[ns] descriptive no significant level of 5% probability; *Significant at 5% probability, ** Significant at 1% probability; *** Significant at 0.1% probability.

Table 3. Deviations values of statistical test and significance levels from the Experiment evaluating the effect of Geneticin as selective agents on indirectly organogenesis of clone BRS07-01, after 30 days.

Variation Sources	Calllus formation	Soots formation	Oxidation frequency	Antocyanin frequency
Trat	D=26,92 <0,001***	D=0,492 0,483^{ns}	D=120,78 <0,001***	D=2,92 0,087^{ns}

^{ns} descriptive no significant level of 5% probability; ** Significant at 1% probability; *** Significant at 0.1% probability;

Table 4. Deviations values of statistical test and significance levels from the Experiment evaluating the effect of Geneticin as selective agents on indirectly organogenesis of clone BRS07-01, after 90 days.

Variation Sources	Calllus formation	Soots formation	Oxidation frequency	Antocyanin frequency
Trat	D=98,06 <0,001***	D=198,47 <0,001***	D=6,28 0,012*	D=42,053 <0,001***

^{ns} descriptive no significant level of 5% probability; * Significant at 5% probability, ** Significant at 1% probability; *** Significant at 0.1% probability;