MARIA LUIZA PEIXOTO DE OLIVEIRA

MORFOGÊNESE *IN VITRO* E TRANSFORMAÇÃO GENÉTICA DE CITROS MEDIADA POR *AGROBACTERIUM TUMEFACIENS*

Tese apresentada à Universidade Federal de Viçosa, como parte das exigências do Programa de Pós-Graduação em Fisiologia Vegetal, para obtenção do título de *Doctor Scientiae*.

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Prof. Fernando Luiz Finger
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“A luta é indispensável para realizar as metas da alma, ou seja, lutar é saudável quando se constrói a felicidade.”
Roberto Shinyashiki

À minha família pelo apoio, amor, carinho e dedicação:

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Ao meu marido, Juliano de Souza Gomes.

À minha amada filha, Isabella Peixoto Gomes.

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BIOGRAFIA

MARIA LUIZA PEIXOTO DE OLIVEIRA, filha de Luiz Carlos Peixoto de Oliveira e Maria do Carmo Carvalho Peixoto, nasceu em Santa Cruz do Rio Pardo, SP, no dia 29 de março de 1977.

Em fevereiro de 1997, ingressou na Universidade Federal de Viçosa – MG, graduando-se em Ciências Biológicas em 2002.

Bolsista do Programa Especial de Treinamento do Curso de Ciências Biológicas da UFV, desde Novembro de 1997 até março de 2002.

No período de janeiro de 1998 a abril de 2002, desenvolveu trabalhos de iniciação científica no Laboratório de Biologia Molecular de Plantas, Instituto de Biotecnologia Aplicada à Agropecuária- BIOAGRO.

Em abril de 2002, iniciou o Mestrado em Genética e Melhoramento. Na Universidade Federal de Viçosa, defendendo tese em fevereiro de 2004.

Em Março de 2004, iniciou o Doutorado em Fisiologia Vegetal, na Universidade Federal de Viçosa, defendendo tese em Abril de 2008.

No período de janeiro de 2007 a fevereiro de 2008, esteve no Departamento de Ciências Horticulturais da Universidade da Flórida, Gainesville- FL, EUA, desenvolvendo parte da tese de doutorado de acordo com o programa de Doutorado-Sanduíche.

CONTEÚDO

RESUMO

OLIVEIRA, Maria Luiza Peixoto de, D.Sc., Universidade Federal de Viçosa, abril de 2008. **Morfogênese *in vitro* e transformação genética de citros mediada por *Agrobacterium tumefaciens*.** Orientador: Wagner Campos Otoni. Co-orientadores: Márcio Gilberto Cardoso Costa e Fernando Luiz Finger.

Os protocolos utilizados para transformação genética de citros têm resultado numa baixa eficiência de transformação, com obtenção de pequeno número de plantas transgênicas, além de utilizarem material juvenil como fonte de explantes necessitando de um longo período para frutificação e conseguinte avaliação de uma característica de interesse introduzida. Um protocolo ideal para a transformação de citros seria baseado na utilização de tecido adulto como fonte de explantes, que reduziria o longo período de juvenilidade, e abreviria o tempo para a análise da característica transgênica incorporada. O objetivo inicial do presente trabalho foi o estabelecimento de um sistema de regeneração *in vitro* a partir de segmentos internodais de plantas adultas de três variedades de laranja doce (*Citrus sinensis* L. Osb.) e limão Cravo (*Citrus limonia* Osb.), visando à otimização da metodologia de transformação. Para a indução de organogênese, investigamos diferentes meios de cultura associados a reguladores de crescimentos (BAP e ANA) e antibióticos β-lactâmicos (timentim, cefotaxima, meropenen e augmentina), utilizados na supressão do crescimento bacteriano e na resposta morfogênica de tecidos adultos de *C. sinensis* e *C. limonia*. Demonstrou-se que a indução *in vitro* de brotações em tecidos

adultos de citros foi afetada pelo genótipo, reguladores de crescimento, formulação do meio e a pela incorporação de antibióticos no meio de cultura. Além disso, foram adequados protocolo otimizados para a transformação genética de segmentos de epicótilo e cotilédones imaturos via *Agrobcaterium tumefaciens*. Para a otimização do protocolo envolvendo explantes provenientes de segmentos de epicótilo, alguns fatores foram investigados, como; o uso de sonicação, infiltração a vácuo e sonicação associada com infiltração a vácuo, comparando-se ao método tradicional de transformação envolvendo *Agrobacterium* (co-cultivo ou imersão na solução bacteriana) durante o co-cultivo de explantes de laranja doce 'Pineapple', e citrumelo 'Swuingle'. A utilização de sonicação por 2 segundos, seguidos por 10 minutos de infiltração a vácuo, teve efeito positivo na eficiência de transformação (8.4%) para laranja 'Pineapple'. Para citrumelo 'Swuingle' a eficiência de transformação também foi aumentada com a combinação de sonicação e infiltração a vácuo (9,6%), mas não foi suficiente para obter uma percentagem maior que a utilização somente da metodologia tradicional de co-cultivo, com eficiência de transformação de 11,2%. Finalmente, estudou-se fatores que pudessem influenciar o processo de indução da morfogênese e a eficiência de transformação em cotilédones imaturos de grapefruit 'Duncan'. Para a indução da organogênese a combinação de 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} AIA promoveu a maior freqüência de cotilédones produzindo brotações (96%) e número de brotações por explante (5,8), utilizando explantes cultivados dorsalmente em contato com o meio de cultura por 3 semanas no escuro, seguido por mais 3 semanas em regime de 16/8 horas (luz/ escuro). Os parâmetros analisados para aumentar a eficiência de transformação mediada por *Agrobacterium* resultaram em uma alta eficiência de transformação obtida quando os expantes foram submetidos a 15 minutos de infiltração a vácuo em presença da Agrobacterium (OD_{600nm} 0,5), co-cultivados por 3 dias em meio contendo 100 μM de acetoseringona e transferidos para meio de seleção, constituído do meio MS contendo 30 mg l^{-1} de canamicina, 12,5 mg l^{-1} de meropenen, 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} AIA.

ABSTRACT

OLIVEIRA, Maria Luiza Peixoto de, D.Sc., Universidade Federal de Viçosa, April of 2008. **Morphogenesis *in vitro* and *Agrobacterium tumefaciens*-mediated genetic transformation of citrus.** Adviser: Wagner Campos Otoni. Co-advisers: Márcio Gilberto Cardoso Costa and Fernando Luiz Finger.

Current protocols used for citrus genetic transformation have resulted in a low number of transgenic plants, furthermore, it used juvenile material which source of explants. Plants regenerated from these sources of explants have long juvenile periods for initial fruit production and are necessary many years for evaluation of the introduced characteristics of interest in the target plant. A reliable and ideal protocol for citrus plant transformation would be based on the use of mature tissue as sources of explant, because the juvenility problem could be lessened and the transgenic trait could be analyzed in a relatively short period of time. Therefore, the initial objective of the present work was to establish promotive conditions for a reliable *in vitro* regeneration system from internodal segments of mature tissue of sweet orange and rangpur lime to further adequate an efficient and reproducible transformation methodology. For organogenesis induction, we investigated the effects of different culture media associated, growth regulator (BAP and NAA) and β-lactams antibiotics, (timentim, cefotaxime, meropenen and augmentin) on the morphogenic response from mature tissue of *Citrus sinensis* and *C. limonia*. We have demonstrated that *in vitro* shoot induction from mature tissue of Citrus was affected

mainly by the genotype, growth regulators, media formulation and antibiotics incorporated to the culture medium. Furthemore, an improved protocol for genetic transformation of epicotyl and immature cotyledons were developed via Agrobacteirum tumefaciens. For protocol involving epicotyl explants some factors were investigated, such as: the use of sonication, vacuum infiltration and sonication in association with vacuum infiltration, comparing with conventional *Agrobacterium*-mediated transformation method ('dipping' method) during co-cultivation of 'Pineapple' sweet orange (*Citrus sinensis* L. Osbeck) and citrumelo 'Swingle' (*Citrus paradisi* Macf. X *Poncirus trifoliate* L. Raf.) explants. The use of sonication for 2 seconds, followed by 10 minutes of vacuum infiltration had a positive effect on putative transgenic efficiency, resulting in the highest transformation efficiency (8.4%) for 'Pineapple' sweet orange. For citrumelo 'Swingle' the transformation efficiency also was enhanced with the combination of sonication and vacuum infiltration (9.6%), however power than the highest scores reached by ordinary co-cultivation protocol, which rended a transformation efficiency of 11.2%. Finally, we studied factors that might influence morphogenesis and *Agrobacterium*-mediated transformation efficiency of immature cotyledons from 'Duncan' grapefruit . For organogenesis induction, the combination of 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA led to the highest frequency of cotyledons forming shoots (96%) and number of shoots per explant (5.8), using explants cultured upside down for 3 weeks in the darkness, followed by 3 weeks in 16/8 h (light/dark) regime. Thus, optimization of *Agrobacterium*-mediated parameters showed that the highest transformation efficiency was achieved when explants were submitted to vacuum infiltration (15 min) in presence of *agrobacteria* (OD_{600nm} 0.5); co-cultivation for 3-days on 100 μM acetoseryngone-supplemented medium; and finally transferred to a selective MS-based medium, added with 30 mg l^{-1} kanamycin, 12.5 mg l^{-1} de Meropenen®, 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA.

INTRODUÇÃO GERAL

As plantas cítricas são originárias das regiões úmidas tropicais e subtropicais do continente asiático, podendo ser cultivadas do Equador até latitudes de 44^{o} norte ou sul (Simão, 1998). O mercado mundial de cítricos apresenta uma forte concentração da produção em dois países: Estados Unidos, nos estados da Flórida e Califórnia, e no Brasil em São Paulo. Juntas, essas regiões respondem por 40 % da produção mundial. Outros países de destaque são China, Espanha, México, Índia e Itália. O Brasil é o maior produtor e exportador de suco de laranja sendo os principais importadores de suco brasileiro a Comunidade Européia (62%), os Estados Unidos (20,5 %) e o Japão (9,2%) (Abecitrus, 2008).

Apesar de o Brasil ocupar lugar de destaque no panorama mundial, houve redução na produção nas últimas safras. Os fatores relacionados à essa diminuição da produção citrícola, merecem destaque as condições climáticas que não favoreceram a produção nas safras anteriores, baixos preços relacionados pelo enfraquecimento do dólar, erradicação de pomares pouco produtivos e problemas fitossanitários

A citricultura brasileira está seriamente comprometida devido à uma nova doença detectada em pomares localizados nas regiões sudoeste de Minas Gerais e norte de São Paulo, denominada morte súbita dos citros (MSC), vem afetando plantas enxertadas sobre limão 'Cravo' (Müller & De Negri, 2001), além das perdas em decorrência do declínio e da gomose do *Phytophthora*. Com relação às copas, principalmente as laranjas doces têm se apresentado igualmente suscetíveis ao cancro

cítrico (*Xanthomonas axonopodis* pv. *citri*) e à clorose variegada dos citros –CVC (*Xylella fastidiosa*) e, mais recentemente, ao 'greening', que são doenças causadas por bactérias sendo que as duas primeiras têm sido as grandes responsáveis por perdas significativas na citricultura nos últimos anos (Feichtenberger, 2000).

Apesar de sua importância econômica no cenário nacional, a citricultura encontra-se bastante vulnerável, face à sua restrita variabilidade genética das variedades porta-enxerto e copa. Os riscos de surgimento de fatores adversos, a exemplo do que se deu no passado com o surgimento da gomose de *Phytophthora* e da tristeza dos citros, em decorrência das variedades copas estarem alicerçadas, respectivamente, nos porta-enxertos laranjeiras 'Caipira' [*C. sinensis* (L.) Osbeck] e 'Azeda' (*C. aurantium* L.). Hoje a base de sustentação da cultura está no porta-enxerto limoeiro 'Cravo' e, sendo assim, a citricultura nacional continua a correr os riscos do surgimento de novos fatores adversos. No que concerne às variedades-copa, apesar do predomínio da laranjeira 'Pêra' (*C. sinensis*), particularmente no Nordeste e Norte do País, outras variedades vêm sendo exploradas, a exemplo da 'Valência' (*C. sinensis*), cujos plantios têm apresentado grande impulso no Estado de São Paulo (Pio et al., 2005), onde se concentram, atualmente, cerca 80% dos pomares cítricos brasileiros

Uma das medidas cabíveis para minimizar os problemas fitossanitários dos citros é o melhoramento genético, desenvolvendo novas variedades tolerantes e, ou, resistentes a pragas e doenças. Entretanto, os métodos tradicionais de melhoramento genético de citros enfrentam uma série de barreiras impostas pela biologia reprodutiva do gênero que dificultam a sua aplicação, dentre elas o longo período juvenil, poliembrionia nucelar, elevada heterozigose, auto-incompatibilidade e esterilidade sexual (Koller, 1994). Devido aos problemas para a aplicação dos métodos convencionais, apesar destes serem de extrema importância para o desenvolvimento da citricultura mundial, há necessidade de métodos complementares para que os avanços ocorram mais rapidamente.

Os problemas associados ao melhoramento das espécies cítricas podem ser superados com a incorporação de técnicas biotecnológicas, a exemplo da cultura de tecidos, genética molecular, fusão de protoplastos, transformação genética, entre outras, permitindo, portanto, a facilitação e a utilização da variedade disponível (Grosser et al., 1996). Dentre estas técnicas, a transformação genética vem mostrando ter um grande potencial, por possibilitar a introdução de genes que

modificam características de interesse agronômico, mantendo-se as características da variedade e evitando a transferência de características deletérias (Peña et al., 1995a).

A transformação genética, mediante a introdução de um único gene em determinado cultivar de *Citrus*, permite produzir rapidamente uma variedade modificada com características específicas (Bond & Roose, 1998). Para tanto, são necessários determinados requisitos para o sucesso na obtenção de plantas transgênicas, tais como a existência prévia de uma metodologia eficiente de propagação *in vitro* que permita a obtenção de plantas (Brasileiro & Dusi, 1999), bem como um sistema de transformação genética compatível, que assegure a introdução de genes com eficiência (Perez- Molphe-Balch & Ochoa-Alejo, 1998).

Os trabalhos de cultivo *in vitro*, em sua maioria, têm utilizado material juvenil como explante, em virtude do baixo nível de contaminação e do elevado potencial morfogênico (Barceló-Muõz et al., 1999). Entretanto, em algumas espécies, principalmente frutíferas, o material juvenil apresenta características agronômicas indesejáveis para a produção de mudas e melhoramento genético. Dentre estas características, destacam-se a presença de espinhos (Hartman et al., 1990), excessivo crescimento vegetativo e o longo período para iniciar o florescimento e frutificação (Ruaud & Pâques, 1995).

O cultivo *in vitro* de espécies frutíferas e ornamentais utilizando tecidos adultos não é frequentemente realizado, em virtude, principalmente, do alto nível de contaminação (Drew, 1988), da redução ou perda da capacidade morfogênica, que está relacionada com a repressão ou inativação progressiva da atividade gênica no desenvolvimento vegetal (Bonga, 1982), e do baixo nível de enraizamento de brotações obtidas (Moore et al., 1992). Mesmo assim, o cultivo *in vitro* de tecidos meristemáticos de plantas adultas tem sido relatado em algumas espécies como *Quercus rubus* L. (Vieitez et al., 1985), *Coffea arabica* L. (Londoño-Ramirez & Orozco-Castaño, 1986), *Carica papaya* L. (Drew, 1988), *Fraxinus ornus* L. (Arrilaga et al., 1992), *Persea americana* Mill (Barceló-Munõz et al., 1999) e *Cyclamen persicum* Mill (Karam & Al-Majathoub, 2000). Um dos poucos relatos de indução de gemas adventícias a partir de tecidos não meristemáticos de plantas adultas tem sido em citros, onde se utilizaram segmentos internodais e nodais de plantas mantidas em casa-de-vegetação (Cervera et al., 1998b; Al-khayri et al., 2001; Al Bahrany, 2002; Almeida et al., 2003; Kobayashi et al., 2003).

Os trabalhos de transformação genética em espécies cítricas iniciaram-se no final da década de 1980 e início da década de 1990 (Kobayashi & Uchimiya, 1989; Hikada et al.,1990; Vardi et al. (1990); Moore et al.,1992). Desde então, diferentes espécies de *Citrus* e gêneros afins já foram utilizadas em trabalhos de transformação genética, destacando-se as laranjeiras doces 'Pineapple' (Peña et aI., 1995b; Cervera et aI., 1998b), 'Washington Navel' (Bond & Roose, 1998) e 'ltaborai' (Fleming et aI., 2000), limeira ácida [*C. aurantiifolia* (Christm.) Swingle] 'Galego' (Peña et aI., 1997; Pérez-Molphe-Balch & Ochoa-Alejo, 1998), laranjeira 'Azeda' (Gutiérrez-E et aI., 1997), citrange 'Carrizo' (Moore et aI., 1992; Peña et aI., 1995a; Cervera et aI., 1998a), *P. trifoliata* (Kaneyoshí et aI., 1994) e pomeleiro 'Duncan' (*C. paradisi*) (Luth & Moore, 1999; Costa et al., 2002). Contudo, a transformação genética em citros tem sido usualmente limitada a tecidos juvenis e plantas obtidas a partir dessas fontes de explantes necessitam de muitos anos para que as características de interesse introduzidas nas plantas transgênicas sejam avaliadas.

Cervera et al. (1998), Peña et al. (2001) e Almeida et al. (2003) relataram o desenvolvimento de um sistema de transformação de citros via *Agrobacterium tumefaciens* utilizando explantes derivados de tecidos adultos. As plantas transgênicas obtidas por Cervera et al. (1998) floresceram e frutificaram 14 meses após transferência para casa-de-vegetação, período de tempo necessário para as plantas maduras atingirem vigor e tamanho adequado para o florescimento e frutificação. Resultados semelhantes foram obtidos por Peña et al. (2001) utilizando construções com os genes LFY e AP1, onde obtiveram plantas transgênicas de citrange 'Carrizo' florescendo 13 a 16 meses após a sua transferência para casa-de-vegetação.

Em *Citrus,* existem diversos protocolos de propagação *in vitro* e transformação genética com formação de plantas, mas as freqüências de transformação não reproduzem resultados satisfatórios para alguns cultivares de laranja e gêneros relacionados, por exemplo, a laranja doce. A baixa eficiência em transformação de *Citrus* está aliada ao desenvolvimento de gemas não transformadas e a dificuldade de enraizamento de gemas transgênicas (Peña et al. 1995a; Gutiérrez-E. et al. 1997; Yang et al. 2000).

Apesar dessas dificuldades, o estabelecimento de um sistema eficiente de propagação *in vitro* e transformação genética no desenvolvimento de variedades-porta-enxerto e copa constitui uma dos objetivos deste projeto, o que também poderá

contribuir em relação às aplicações práticas de resultados obtidos nos Projetos Genoma da *Xylella* e *Xanthomonas*, bactérias causadoras da clorose variegada dos citros (CVC) e do cancro cítrico, respectivamente.

Diante disto, os objetivos do presente estudo foram estabelecer condições adequadas de cultivo *in vitro* de material adulto proveniente de segmentos internodais para três cultivares de laranja doce ('Pêra', 'Valência' e 'Bahia') como também para limão 'Cravo', e estudar diferentes metodologias ainda não descritas na literatura, como utilização de sonicação e vácuo durante o processo de incubação do material vegetal, proveniente de material juvenil (epicótilo e cotilédones), na suspensão bacteriana visando aumentar a eficiência de transformação em espécies cítricas.

Na primeira parte do estudo, investigou-se o efeito de meios de cultura associados com diferentes concentrações de reguladores de crescimento e também comparou-se a eficiência de diferentes formulações de antibióticos: ticarcilina (timentim), cefotaxima, meropenem e augmentina durante o processo de contenção do crescimento de bactérias exógenas e também se comparou o efeito desses antibióticos na resposta morfogênicas dos cultivares acima citados. Na segunda parte do estudo, objetivou-se a otimização de alguns fatores para aumentar a eficiência de transformação via *Agrobacterium tumefaciens* para 'Pineapple' e citrumelo 'Swingle', examinando-se o uso de ultrasom (SAAT) e de vácuo comparando-os com o método clássico (imersão em *Agrobacterium*). Finalmente, na terceira, a morfogênese *in vitro* em segmentos cotiledonares de 'grafefruit 'Duncan' foi avaliada e um protocolo otimizado para transformação dessa espécies foi desenvolvido, baseado na expressão transiente do gene *gus*, examinado-se cinco fatores relacionados à eficiência de transformação.

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CAPÍTULO I

PLANT REGENERATION OF CITRUS FROM MATURE TISSUE: GENOTYPES DIFFER IN HORMONE REQUIREMENTS AND IN THEIR RESPONSE TO ANTIBIOTICS

ABSTRACT

Plant regeneration from mature tissues of citrus is an absolute requirement for the rapid evaluation of the resulting traits following the genetic transformation experiments. The influence of growth regulators, basal medium formulations, and four β-lactam antibiotics (timentin, cefotaxime, meropenen, and augmentin) on adventitious shoot regeneration in mature internodal segments was compared in three genotypes of *Citrus sinensis* L. Osbeck ('Pêra', 'Bahia', and 'Valência') and one genotype of *Citrus limonia* Osbeck ('Cravo'). The results indicated that the combination of the cytokinin 6-benzylaminopurine (BAP) and the auxin α-naphthaleneacetic acid (NAA) is necessary for effective shoot regeneration, but the optimal balance between these growth regulators is genotype-specific. A higher regeneration frequency was observed when the explants were cultured on Murashige and Skoog (MS) and Murashige and Tucker (MT) media as compared to Woody Plant medium (WPM), although those explants cultured on WPM produced larger shoots. All β-lactam antibiotics inhibited bacterial growth on culture medium at

higher concentrations, but their effects on shoot organogenesis depended on type and concentration of the antibiotics and genotype analyzed. Cefotaxime at 500 mg l^{-1} enhanced shoot regeneration in 'Pêra' and 'Valência'. In 'Cravo', most of the antibiotics and their concentrations tested negatively affected *in vitro* morphogenesis. These differences in organogenic responses clearly demonstrate that, as well as it has been reported for juvenile explants, the optimal conditions for regeneration of explants from mature citrus plants must be established for each genotype.

***Key words*:** Antibiotics, citrus, mature tissues, organogenesis.

INTRODUCTION

Commercial citrus plants are faced by a number of pests, diseases and abiotic stresses which limit their production worldwide (Whiteside *et al.* 1993). In addition, fruit quality needs to be continually improved to meet the consumers demands. A long-term solution to these problems is the production of citrus varieties that are genetically tolerant to these environmental challenges and/or producing fruits of increased organoleptic and nutritive desirability. However, it is not a simple task to achieve using conventional breeding, given the reproductive biology of the genus *Citrus*, the long-term nature of tree breeding, and the complex hybrid nature of commercially acceptable citrus types. Therefore, genetic transformation is an essential tool to overcome these limitations and to accelerate the improvement of acceptable citrus varieties that are deficient in one or a few characteristics.

Several methods have been described in the literature for genetic transformation of citrus, but the most effective methods so far are those using *Agrobacterium*-mediated transformation of juvenile material involving zygotic embryos, hypocotyl, epicotyl, and cotyledons (Hidaka *et al.* 1990, Moore *et al.* 1992, Peña *et al.* 1995a, Peña *et al.* 1995b, Peña 1997, Gutiérrez-E. *et al.* 1997, Bond and Roose 1998, Cervera *et al.* 1998, Perez-Molphe-Balch and Ochoa-Alejo 1998, Fleming *et al.* 2000, Al-Bahrany 2002, Costa *et al.* 2002). Plants regenerated from these sources of explants have long juvenile periods for initial fruit production and many years are necessary before the evaluation of the horticultural and commercial

traits introduced in the transgenic plants. A reliable and ideal protocol for citrus plant transformation would be based on the use of mature tissue as explant source, because the juvenility problem could be circumvented and the introduced traits could be analyzed in a relatively short period of time (Cervera *et al.* 2000, Peña *et al.* 2001, Almeida *et al.* 2003, Cervera *et al.* 2007, Peña *et al.* 2007, Rodríguez *et al.* 2008).

For successful genetic transformation of mature tissue, the first step is the establishment of an efficient plant regeneration system, since *in vitro* culture of fruit species using mature tissue as explants is still far from routine (Almeida *et al.* 2003, Rodríguez *et al.* 2008). Reasons for this include the relatively low responsiveness of woody plants to exogenous growth regulators, and the failure of standard surface sterilization techniques (Cervera *et al.* 2007). A growth medium with the adequate plant growth regulator and optimal mineral conditions increase the possibility of success for the protocol of plant transformation.

The inability to adequately control *in vitro* contamination, mainly by endophytic bacteria, is the primary reason for failure of plant regeneration protocols using mature tissues as explants. It could be advantageous, therefore, the supplementation of antibiotics in the culture medium for complete elimination of the bacterial contaminants. However, a careful evaluation of their effects on plant regeneration must be also carried out, since several reports have shown that antibiotics which are commonly used to eliminate bacteria from plant tissues could positively or negatively affect *in vitro* morphogenesis (Eapen and George 1990, Chang and Schmidt 1991, Lin *et al.* 1995, Nauerby *et al.* 1997, Cheng *et al.* 1998, Costa *et al.* 2000, Tang *et al.* 2005).

β-lactam antibiotics, such as carbenicillin and cefotaxime, are the most commonly used antibiotics in plant transformation protocols, since they have a broad spectrum of activity against bacteria and a low toxicity to eukaryotes (Borrelli *et al.* 1992, Pius *et al.* 1993, Rao *et al.* 1995, Cheng *et al.* 1998, Ling *et al.* 1998, Humara and Ordas 1999, Bhau and Wakhlu 2001, Yu *et al.* 2001). Nevertheless, a number of plants responded negatively to cefotaxime (Yepes and Aldwinckle 1994, Nauerby *et al.* 1997, Ling *et al.* 1998, Ogawa and Mii 2005). Another β-lactam antibiotic commonly utilized to suppress bacteria in plant cell culture is timentin (ticarcillin associated with clavulanic acid) (Vergauwe *et al.* 1996, Ling *et al.* 1998).

Recently, Ogawa and Mii (2005, 2006) and Ying *et al.* (2006) evaluated the usefulness of meropenen, novel β-lactam antibiotics, which are a new generation

carbapenen-based antibiotic. These studies showed that meropenen had little effect on the growth and the morphogenic response of analyzed species, being an alternative antibiotic for the effective suppression of bacteria in genetic transformation protocols. Preliminary studies developed by Ogawa and Mii (2004) showed a high active with meropenen against agrobacteria strain EHA 101 and similar experiment showed similar high in planta bacterial against LBA4404 (Ogawa and Mii 2005)

Little is known about the effects of antibiotics on *in vitro* morphogenesis of woody plants. It has been speculated that some antibiotics act somehow as a regulator of the morphogenic development, and that they can be utilized for improving the *in vitro* response. In this report, we have investigated the response to hormone additions and β-lactams antibiotics of mature internodal segments from different citrus genotypes, including the commercially important citrus cultivar 'Bahia' (Washington navel orange), and a protocol for the regeneration of whole plants is described. To our knowledge, this is the first time that the effect of antibiotics on citrus morphogenesis has been evaluated.

MATERIALS AND METHODS

Plant material

Internodal segments with approximately 1.0-1.5 cm long from greenhouse-grown adult plants of 'Pêra', 'Valência', and 'Bahia' sweet oranges (*Citrus sinensis* L. Osbeck) and 'Cravo' rangpur lime (*Citrus limonia* Osbeck) were used as source of mature explants.

Rejuvenation was applied to facilitate *in vitro* culture of explants from mature tissue, by grafting buds into juvenile rootstocks. These mother plants were drastically pruned to stimulate the sprouting of the basal buds which could keep juvenile characters. These plants were regularly sprayed with the fungicide Benlate at 1% (v/v) to prevent contamination during *in vitro* culture. Vigorous newly elongated lateral branches in a semi-hardened stage were collected. Under aseptic conditions they were surface sterilized in 70% (v/v) ethanol for 2 min, followed by 5% (v/v) commercial solution of sodium hypochlorite (Super Globo®, Brazil) containing 0.1% (v/v) Tween-20 for 20 min, and sequentially rinsed four times in sterile distilled water. Thereafter, internodal explants were isolated, cut horizontally into halves, and placed upside down onto different media to examine the best inductive conditions for *in vitro* adventitious organogenesis as influenced by growth regulators (6-benzylaminopurine - BAP and α-naphtaleneacetic acid - NAA combinations) and different β-lactam antibiotics.

Media, growth regulators and culture conditions

Mature internodal segments from all four cultivars were prepared as described above and placed onto MS medium added with different concentrations of BAP (0.25, 0.50, 1.0 and 2.0 mg dm^{-3}) in combination with NAA (0.0, 0.25, 0.5 and 1.0 mg dm^{-3}), for induction of shoot regeneration. All media were supplemented with 3% sucrose and 6.5% agar (Merck), with pH adjusted to 5.7 prior to autoclaving. The media were poured (25 cm^{-3} aliquots) into sterile 90 X 15 mm Petri dishes (J. Prolab, Brazil). Explants were kept initial incubation in darkness at 26 ± 2 ^{0}C for 30 days and then transferred under the 16/8-h (light/dark), photoperiod 36 μmol m^{-2} s^{-1} light radiation provided by two 20 W white fluorescent tubes (Osram, Brazil). They were subcultured every 10 days to prevent bacteria overgrowth.

For testing the influence of culture media on shoot-bud induction, three formulations were evaluated: MS (Murashige and Skoog 1962), MT (Murashige and Tucker 1969) and WPM (Lloyd and McCown 1980). The best combination of plant growth regulators, associated with the three different genotypes, described in the first set of experiment, was used in media formulation.

Elongated shoots (1-1.5 cm long) were excised and subcultured onto MS medium supplemented with 0.5 mg dm^{-3} NAA for root induction. Therefore, after 60 days on culture medium, rooted shoots were transplanted to plastic cups containing sterile soil, sand and vermiculite (1:1:1, v/v/v) and were placed in illuminated shelves under 24 μmol m^{-2} s^{-1} irradiance, provided by two 20 W white fluorescent tubes (Osram, Brazil).

Effect of antibiotics

To determine the influence of β-lactams antibiotics on shoot regeneration, the best combination of genotype, culture media and growth regulator, obtained in the previous experiments was used in combination with different antibiotics. Four antibiotics were evaluated on their effect upon shoot regeneration: timentin (SmithKline Beecham, Brazil) (300 and 500 mg dm^{-3}), cefotaxime sodium salt (Novafarma, Brazil) (250 and 500 mg dm^{-3}), meropenen trihydrate (ABL, Brazil) (25; 50; 75 and 100 mg dm^{-3}) and augmentin (GlaxoSmithKline, Hungary) (250 and 500 mg dm^{-3}). The antibiotics were dissolved in water, filter-sterilized in 0.2 μm pore

size membranes (Millex, Ireland), and added to the culture medium after autoclaving, throughout cooling process. Explants were incubated on 25 cm^{-3} aliquots of semisolid medium under the same conditions as previously described.

Statistical analysis

A completely randomized design was used, and each experiment was repeated twice. Eleven explants were cultured per plate and 5 plates were used for each treatment. The regeneration frequency and number of shoots per explants were scored after a 60 days period. Statistical analysis were carried out using analysis of variance, and treatments means were separated using Tukey Test (P = 0.05), performed with the software SAEG (Sistema de Análises Estatísticas e Genéticas, Federal University of Viçosa, Brazil),

RESULTS AND DISCUSSION

Medium composition and hormonal effect on adventitious bud induction

The regenerative potential of mature stem segments from different citrus genotypes was assessed in culture media of distinct basal composition and with combinations of the growth regulators BAP and NAA. Plant regeneration via direct and indirect organogenesis was achieved on the surface of the cut zone from cultured internodal explants of all four citrus genotypes tested. Small amount of compact callus arose on the cut surface with two weeks of culture when the segments were incubated in darkness. Shoot buds differentiated from these callus two weeks after transferring the cultures to light condition. Shoots were successfully differentiated (Table 1 and Fig. 1*A*) and shoot induction was statistically significant ($p = 0.05$) as the level of BAP increased in the medium. But the effect of BAP on morphogenesis depended on NAA concentration. Thus, the combination of these cytokinin and auxin in the culture medium is the pre-requisite needed to induce adventitious shoot regeneration in explants of mature citrus plants.

Table 1 – Effect of BAP and NAA on shoot organogenesis from internodal stem segments of Citrus, after 60 days in MS-based culture medium

BAP x NAA (mg dm^{-3})	Number of shoots/explant				Explants forming shoots (%)			
	'Pêra'	'Valência'	'Bahia'	'Cravo'	'Pêra'	'Valência'	'Bahia'	'Cravo'
0.0 x 0.0	0.19^{e}	0.00^{e}	0.09^{e}	0.12^{e}	9.1^{e}	0.00^{c}	5.46^{f}	10.90^{e}
0.25 x 0.0	1.00^{cd}	0.40^{cde}	0.94^{de}	1.10^{bcde}	29.10^{cd}	16.36^{bc}	18.18^{ef}	41.81^{cd}
0.25 x 0.25	0.60^{de}	0.27^{de}	0.76^{cde}	0.74^{cde}	16.40^{de}	14.54^{bc}	21.82^{def}	30.90^{cde}
0.50 x 0.0	1.18^{bcd}	1.07^{abcde}	1.10^{bcde}	2.37^{ab}	47.26^{abc}	36.36^{ab}	54.54^{abc}	65.45^{ab}
0.50 x 0.25	1.27^{bcd}	0.98^{abcde}	1.23^{abcd}	3.05^{a}	56.36^{ab}	30.90^{ab}	45.45^{bcd}	72.72^{a}
0.50 x 0.50	0.94^{cd}	0.88^{abcde}	0.90^{bcde}	1.77^{bc}	43.63^{bc}	32.72^{ab}	41.81^{bcde}	58.17^{abc}
1.0 x 0.0	1.39^{bcd}	1.83^{ab}	1.74^{abc}	2.03^{abc}	69.10^{a}	56.36^{a}	70.90^{a}	63.63^{ab}
1.0 x 0.25	1.94^{ab}	2.12^{a}	2.23^{a}	1.99^{bc}	67.27^{a}	54.54^{a}	65.44^{ab}	60.00^{ab}
1.0 x 0.50	2.46^{a}	1.99^{a}	1.94^{ab}	1.47^{bcd}	61.81^{ab}	52.72^{a}	63.63^{abc}	58.17^{abc}
1.0 x 1.0	1.05^{cd}	0.60^{bcde}	0.88^{bcde}	0.67^{cde}	52.72^{ab}	51.51^{a}	40.00^{cde}	29.08^{de}
2.0 x 0.0	1.25^{bcd}	1.28^{abcd}	1.08^{bcde}	1.01^{bcd}	56.36^{ab}	56.36^{a}	60.00^{abc}	38.18^{cde}
2.0 x 0.25	1.63^{abc}	1.45^{abcd}	1.38^{abcd}	0.70^{cde}	58.17^{ab}	50.36^{a}	56.36^{abc}	32.72^{cde}
2.0 x 0.50	1.56^{bc}	1.36^{abcd}	1.12^{abcd}	0.80^{cde}	56.60^{ab}	45.45^{a}	52.72^{abc}	29.08^{de}
2.0 x 1.0	2.05^{ab}	1.43^{abc}	1.17^{abcd}	0.54^{de}	58.18^{ab}	43.63^{a}	50.90^{abc}	25.45^{de}

Means followed by the same letter within a column do not differ significantly by Tukey test (p = 0.05).

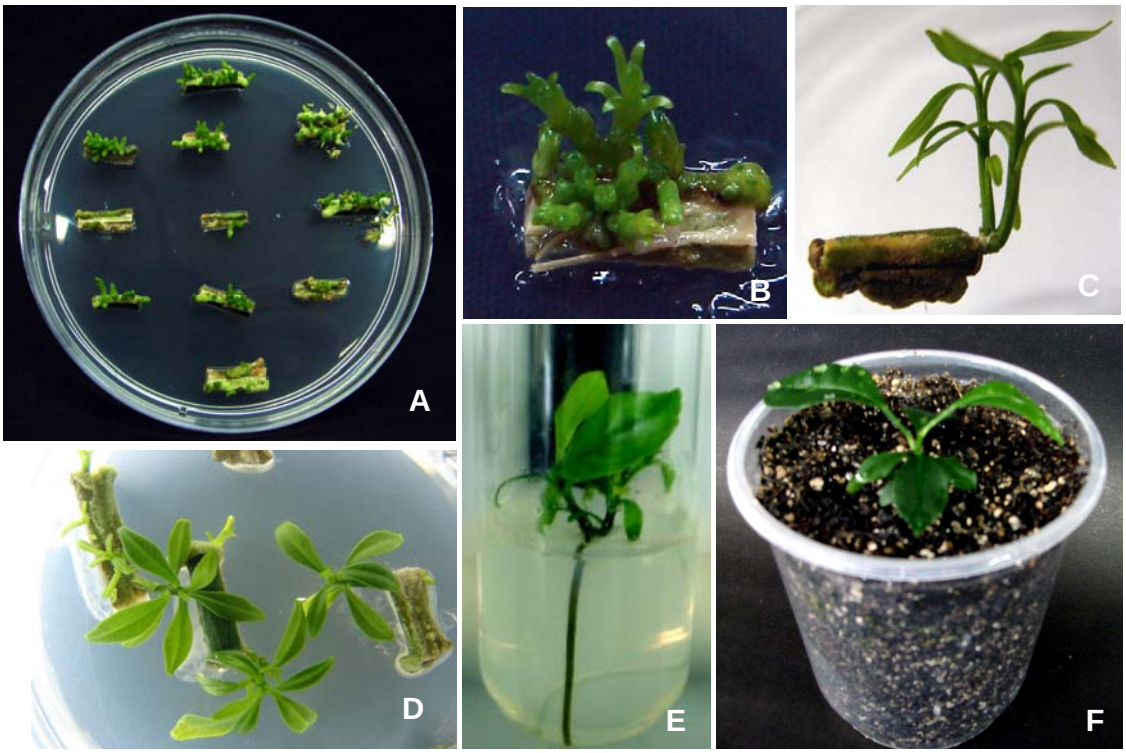

Figure 1 – Adventitious bud formation from internodal stem segments of 'Cravo'. (*A*) multiple adventitious buds after 45 days in culture; (*B*) detail of an internodal segment cultured in MS medium supplemented with 0.50 mg dm^{-3} BAP and 0.25 mg dm^{-3} NAA; (*C*) internodal segments cultured in WPM medium after 45 days in culture; (*D*) explants placed on medium with low antibiotic concentration showing endophytic bacteria; (*E*) rooted plantlet after 60 days in rooting medium; (*F*) rooted shoot placed on soil to acclimatization in laboratory condition.

The optimal hormone addenda varied among citrus genotypes: the highest regeneration efficiency and shoots per explant were obtained in response to 1 mg dm^{-3} BAP and 0.50 mg dm^{-3} NAA in 'Pêra'; 1 mg dm^{-3} BAP and 0.25 mg dm^{-3} NAA in 'Bahia' and 'Valência'; and 0.50 mg dm^{-3} BAP and 0.25 mg dm^{-3} NAA for 'Cravo'. 'Cravo' rangpur lime showed higher shoot regeneration percentage (72.72%) and number of regenerated shoots per explant (3.05), followed by 'Pêra' (61.81% and 2.46 shoots/explant) and 'Bahia' (65.44% and 2.23 shoots/explant). The lowest shoot regeneration capacity was observed in 'Valência' sweet orange (54.54% and 2.12 shoots/explant). For this genotype, no bud formation was observed when BAP and

NAA were omitted from the medium, as well as low regeneration frequencies and number of shoots per explant were obtained for the other citrus genotypes assessed.

The ability to generate shoots in tissue culture is a genetically controlled trait that may be modified by the manipulation of growth regulators combined with ideal level of cytokinin and/or auxin. The endogenous balance between cytokinins and auxins is crucial for *in vitro* culture establishment. Thus, the addition of growth regulator to the culture medium must be investigated since explants from juvenile or mature tissues respond differently to a certain plant growth regulator. It has been previously reported that differently of most juvenile explants, explants from mature citrus plants require a combination of BAP and NAA in culture medium for effective shoot regeneration (Almeida *et al.* 2003, Rodríguez *et al.* 2008). The present results corroborate with this finding and further demonstrate that the optimal balance between BAP and NAA in the culture medium is genotype-specific.

After set up the optimal hormone requirements for each genotype, it was investigated if different formulations of basal media could affect the shoot organogenesis potential of mature tissues. The composition of the culture media used for citrus tissue culture is usually based on the nutrients and vitamins of MS and MT, although WPM has been successfully used for tissue culture of recalcitrant woody species. No significant difference as to shoot induction was observed when explants were cultured on MS or MT medium, irrespective of genotype analyzed (Fig. 2). A general trend observed was that MS and MT media stimulated more morphogenesis as compared to WPM medium; however, those explants cultured in WPM produced larger shoots than those cultured on MS and MT basal media (Figure 1b and 1c). Such observation has been previously reported for citrus (Kobayashi *et al.* 2003, Cervera *et al.* 2007). It has been suggested that the lower concentration of nitrogen and potassium in WPM medium could account for these observed differences in shoot size (Kobayashi *et al.* 2003).

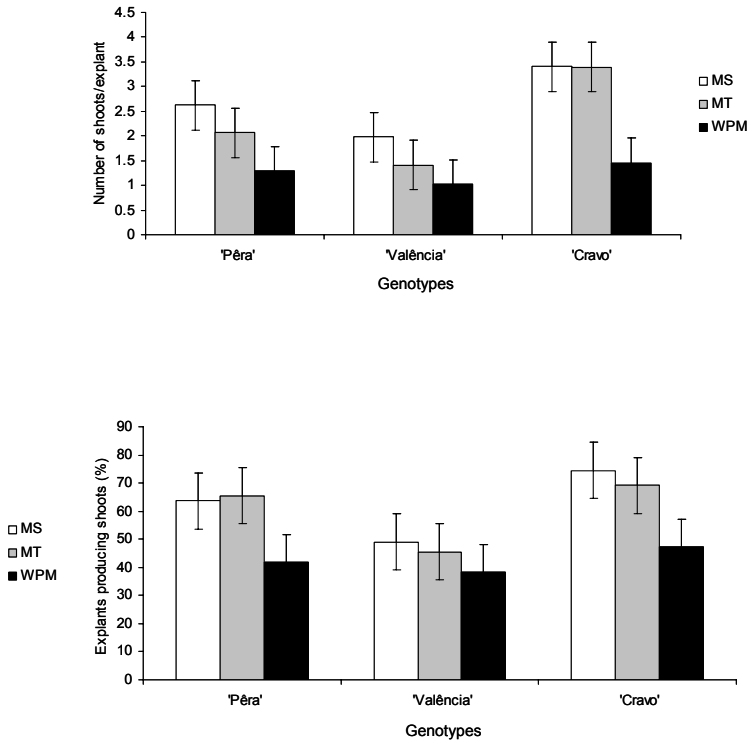

Figure 2 – Influence of the culture medium on shoot organogenesis from mature internodal segments of three citrus genotypes, after 60 days of *in vitro* culture. The vertical bars correspond to the standard deviations of the means.

Effects of β-lactam antibiotics on shoot formation

Bacterial contamination during *in vitro* culture of mature internodal segments from greenhouse-grown mother plants is in most cases responsible for no shoot formation ability observed in explants cultured on antibiotic-free medium. Therefore, the effects of four β-lactam antibiotics on bacterial decontamination as well as on shoot regeneration potential of mature citrus tissues were evaluated in the present study. The degree of decontamination and morphogenesis activation depended on the genotype and type and concentration of antibiotic utilized. Table 2 summarizes the effect of meropenen, timentin, cefotaxime and augmentin on shoot formation from mature internodal segments of citrus species.

Table 2 – Influence of different antibiotics on shoot organogenesis from mature internodal segments of Citrus varieties, cultured on MS medium for 60 days

Antibiotic concentration (mg dm^{-3})	Number of shoots/explant			% Explants producing shoots		
	'Pêra'	'Valência'	'Cravo'	'Pêra'	'Valência'	'Cravo'
Control	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{e}	0.00^{f}	0.00^{e}
Meropenen						
25	0.84^{de}	0.58^{de}	1.09^{cde}	34.42^{abcd}	14.44^{ef}	39.80^{d}
50	1.40^{bcd}	1.60^{abcd}	1.80^{bcd}	52.80^{abc}	54.63^{bcd}	63.79^{abcd}
75	1.56^{bcd}	1.57^{abcd}	1.68^{bcd}	49.00^{abcd}	65.58^{ab}	65.82^{abc}
100	0.60^{de}	1.20^{bcd}	0.84^{de}	21.65^{cde}	36.20^{cde}	49.20^{bcd}
Timentin						
300	0.38^{de}	1.36^{abcd}	1.23^{cd}	25.47^{bcde}	54.80^{bcd}	43.60^{cd}
500	2.20^{bc}	2.02^{ab}	2.40^{ab}	56.39^{ab}	60.18^{abc}	69.38^{ab}
Cefotaxime						
250	0.75^{de}	1.40^{abcd}	1.09^{cde}	19.80^{de}	54.60^{bcd}	43.41^{cd}
500	4.03^{a}	2.45^{a}	3.04^{a}	58.46^{a}	83.80^{a}	76.64^{a}
Augmentin						
250	1.07^{cde}	0.62^{de}	0.99^{cde}	54.60^{ab}	27.17^{def}	43.58^{cd}
500	2.41^{b}	1.63^{abcd}	2.05^{abc}	52.80^{abc}	56.60^{abc}	56.60^{abcd}

Means followed by the same letter within a column do not differ significantly by Tukey test ($p = 0.05$).
Control treatment consists of the regeneration medium without antibiotics; note the absence of regeneration, because bacteria overgrowth was observed on explants inhibiting the shoot differentiation.

In general, the lowest concentrations of all antibiotic tested were not able to suppress bacterial overgrowth, including meropenen at 25 mg dm^{-3}, timentin at 300 mg dm^{-3}, and augmentin at 250 mg dm^{-3}. In these concentrations of the antibiotics, the extensive bacteria growth caused a necrotic process in many explants, and consequently a negative influence on shoot formation. In explants cultured on 250 mg dm^{-3} augmentin, bacteria growth was observed after 7 days (Fig. 1*D*). At higher concentrations, all antibiotics inhibited bacterial growth, with additional positive or negative effect on shoot formation depending on type and concentration used and genotype analyzed.

The best *in vitro* responses of the mature explants were obtained in 500 mg dm^{-3} cefotaxime, since it had either a promotive effect ('Pêra' and 'Valência') or no negative effect on shoot regeneration ('Cravo'). In 'Pêra', the mean number of shoots per explant increased from 2.46 (Table 1) to 4.03 (Table 2) in this concentration of the antibiotic. In 'Valência', it also increased the shoot formation percentage from 54.54% (Table 1) to 83.8% (Table 2). All other antibiotics and concentrations tested affected negatively shoot regeneration in the genotypes evaluated, with exception of timentin at 500 mg dm^{-3} and augmentin at 500 mg dm^{-3}, which did not inhibit shoot regeneration in 'Pêra' and 'Valência' as compared to the media devoid of antibiotics (Table 1). These results indicated, therefore, that there are genotypic differences in the morphogenic response to β-lactam antibiotics, with 'Cravo' more sensitive to their toxic effects than 'Pêra' and 'Valência'.

The stimulatory effect of the antibiotic cefotaxime on plant morphogenesis has been also observed in wheat (Mathias and Boyd 1986, Borrelli *et al.* 1992), barley (Mathias and Mukasa 1987), *Picea glauca* (Ellis *et al.* 1989), *Eleusine caracana* (Eapen and George 1990), apple (Yepes and Aldwinckle 1994), sorghum (Rao *et al.* 1995), *Pinus pinea* (Humara and Ordas 1999), *Coryphantha elephantides* (Bhau and Wakhlu 2001), maize (Danilova and Dolgikh 2004). Phytotoxic effect has been reported in white spruce (Tsang *et al.* 1989) and apple (James *et al.* 1989, James and Dandekar 1991). Feyissa *et al.* (2007) pointed out that addition of cefotaxime at 200 mg dm^{-3} into the medium was not toxic effects on *H. abyssinica* but cefotaxime at 500 mg dm^{-3} did not affect callus formation either although shoot regeneration was significantly reduced compared to control.

Nakano and Mii (1993) raised some possibilities to explain the mechanism of the stimulatory effect of antibiotics on plant morphogenesis. The antibiotic

compounds may mimic plant growth regulators, but the molecular structure of cefotaxime, a semi-synthetic analog of cephalosporin member of β-lactam group, showed that it did not mimic the activity of auxin, cytokinin or gibberellin. However, their degradation by-products by plant esterases can generate metabolites with plant growth regulator properties (Mathias and Mukasa 1987). These compounds generated by carbenicillin breakdown seem to exhibit phenylacetic acid auxin-like activity stimulating growth and morphogenesis in sunflower cultivar Centennial (Orlikowska *et al.* 1995).

Shoots regenerated from internodal explants of three genotypes cultured on medium containing different antibiotics were transferred to rooting medium (Fig. 1*F*). It was demanded the maintenance of the antibiotics in rooting medium to avoid bacterial resurgence. Thus, none of antibiotics tested were able to completely suppress bacterial contamination.

In conclusion, we have demonstrated in the present study that citrus genotypes differ in hormone requirements and in their response to antibiotics when mature tissues are used as explant source. The results suggest that the combination of BAP and NAA is a requirement to support effective shoot regeneration, but the optimal balance between these growth regulators varies among the genotypes. Furthermore, the use of 500 mg dm^{-3} cefotaxime in the culture medium is advantageous both to control bacterial contamination and to influence positively the morphogenetic process, especially in sweet orange. This protocol might be readly used in genetic transformation of mature internodal segments for incorporation of reporter genes and agronomically important genes by means of *Agrobacterium* infection.

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CAPÍTULO II

HIGH-EFFICIENCY *AGROBACTERIUM*-MEDIATED TRANSFORMATION OF CITRUS VIA SONICATION AND VACUUM INFILTRATION

ABSTRACT

An efficient method to enhance *Agrobacterium* infiltration into epicotyl segments of 'Pineapple' sweet orange [*Citrus sinensis* (L.) Osbeck] and Swingle citrumelo [*Citrus paradisi* Macf. X *Poncirus trifoliata* (L.) Raf.] was developed in order to obtain a high frequency of transformants. The epicotyl portions of etiolated seedlings were cut transversally into segments (0.8-1 cm) and inoculated into *Agrobacterium tumefaciens* strain EHA 101/pGA482GG suspension. The explants were submitted to pulses of ultrasound at 35 W for 2-30 s, vacuum infiltration at 75 in Hg for 5-25 min, or a pulse of ultrasound for 2 s combined with vacuum infiltration at 60 in of Hg for 10 min, and then cocultivated in the dark at 26 ± 2 ^{o}C for 2 days. Subsequently, the explants were transferred to selective shoot regeneration medium. Histochemical GUS assays were performed after 7 days, to measure *uidA* transient expression, or after 45 days to quantify stable transformation. Southern blot of *uidA* gene was used to confirm the integration of the transgenes. The transformation frequencies achieved in this study, 8.4% for 'Pineapple' sweet orange and 11.2% for 'Swingle' citrumelo are the highest ones reported for both cultivars.

INTRODUCTION

Citrus genetic transformation is a tool that has been available to assist breeding programs for the past few decades. However, several citrus types, including the commercially important sweet oranges [*Citrus sinensis* (L.) Osbeck], exhibit low transformation efficiencies, even though improvements in *Agrobacterium*-mediated methodology have been achieved (Peña et al., 1995a; Peña et al., 1995b; Peña et al.,1997; Gutiérrez et al., 1997; Bond and Roose, 1998; Costa et al., 2002; Rodríguez et al., 2008).

A critical step in the development of *Agrobacterium tumefaciens*-mediated transformation procedures is the establishment of adequate conditions for T-DNA delivery into the host cell (Amoah et al., 2001). A range of factors, such as preculture regimes, manipulation of inoculation, and cocultivation conditions have been observed to play a significant role in influencing tissue competence (Peña et al., 1997; Costa et al., 2002).

Adoption of specific wounding methods and use of vacuum infiltration in transformation protocols have been employed to enhance transformation frequencies in several plant species. These methodologies have been described in the literature: examples include wounding during explant preparation (Horsch et al., 1985; Charity et al., 2002); delivery of the bacterium to the target tissue via syringe (Chee et al., 1989); gentle stabbing explants a few times with a sterile hypodermic needle for wounding (Xue et al., 2006); the use of particle gun-mediated micro-wounding prior

to *Agrobacterium*-mediated transformation (Bidney et al., 1992); and the use of ultrasound to enhance transformation rates in plant tissue (Trick and Finer, 1997; Gaba et al., 2006). The exudates of wounded tissue often produce acetosyringone (AS) and α-hydroxyacetosyringone (OH-AS), which induce the entire *vir* regulon in *Agrobacterium* as well as the formation of T-DNA intermediate molecules (Stachel et al., 1985).

Sonication-assisted *Agrobacterium*-mediated transformation (SAAT) is a relatively new technology for introducing *Agrobacterium* into the target cell. SAAT has been shown to provide efficient delivery of T-DNA to cells of a number of plants (Santarém et al., 1998; Tang et al., 2001; Zaragozá et al., 2004; Beranová et al., 2008), especially those that are typically more recalcitrant to *Agrobacterium*-mediated transformation (Trick and Finer, 1997). This method involves subjecting the plant tissue to brief periods of ultrasound in the presence of the *Agrobacterium* (Liu et al., 2006). Plant cells have a hard and thick cell wall and the SAAT treatment produces a large number of small and uniform wounds across the tissue allowing *Agrobacterium* easy access into the target plant cells or tissue. It allows the *Agrobacterium* to travel deeper and more completely throughout the tissue than normal cocultivation will permit (Trick and Finer, 1997; Santarém et al., 1998; Tang, 2001; Liu et al., 2005), thus enhancing the bacteria colonization and infection of the tissue.

Another methodology widely reported to enhance *Agrobacterium* infection is vacuum-infiltration, which has been successfully used to produce transgenic plants of bean (Liu et al., 2005), *Arabidopsis* (Clough and Bent, 1998), banana (Acereto-Escoffié et al., 2005), coffee (Canche-Moo et al., 2006), cotton (Ikram-Ul-Hal, 2004; Leelavathi et al., 2004), kidney bean (Liu et al., 2005), Monterey pine (Charity et al., 2002), and wheat (Cheng et al., 1997; Amoah et al., 2001). This process increases gene transfer efficiency by improving penetration of *Agrobacterium* cells into the plant tissue layers. Association of this method with sonication or other methods of wounding have improved T-DNA delivery into the target tissue (Charity et al., 2002; Liu et al., 2005)

Many studies have investigated transformation protocols for juvenile explants of *Citrus* species (Hidaka et al., 1990; Moore et al., 1992; Peña et al., 1995; Gutiérrez-E. et al., 1997; Peña et al., 1997; Bond and Roose, 1998; Cervera et al., 1998; Perez-Molphe-Balch and Ochoa-Alejo, 1998; Fleming et al., 2000; Costa et

al., 2002; Molinari et al., 2003; Kayim and Koc, 2005; Duan et al., 2007), but there are no reports on the effect of sonication and/or vacuum infiltration to enhance *Agrobacterium* infection in citrus species. Thus, we investigated in the present report the use of sonication, vacuum infiltration, and a combination of the two compared to our conventional *Agrobacterium*-mediated innoculation method ('dipping' method) using 'Pineapple' sweet orange and Swingle citrumelo epicotyl explants.

MATERIALS AND METHODS

Plant material

Etiolated epicotyl segments from *in vitro*-germinated seedlings of 'Pineapple' sweet orange (*Citrus sinensis* L. Osbeck) and Swingle citumelo (*Citrus paradisi* Macf. X *Poncirus trifoliata* L. Raf.) were used as explant source. Swingle is an important rootstock for commercial citrus production in the USA and 'Pineapple' is the Florida's principal midseason sweet orange cultivar showing an excellent texture and juice quality.

Seeds of 'Pineapple' and Swingle were obtained from the University of Florida Citrus Research and Education Center at Lake Alfred, and stored in a refrigerator at 4 ^{o}C. The seeds were peeled and the external coat was removed, and they were surface-sterilized for 1 min in 70 % (v/v) ethanol, and further immersed in a solution containing 2.5 % (v/v) commercial bleach (Ultra Bleach, USA) and 0.1 % (v/v) Tween 20, with slight agitation for 15 min, then rinsed three times with sterile distilled water.

Surface-sterilized seeds were inoculated into 21 x 150 mm tubes containing 12 ml of half-strength MS basal medium (Murashige and Skoog, 1962), supplemented with 50 mg l^{-1} myo-inositol, 25 g l^{-1} sucrose and solidified with 7.0 g l^{-1} agar (PhytoTechnology Laboratories, USA) for germination. The test tubes were capped with polypropylene closures. The cultures were incubated in a growth chamber at 27 ± 2 ^{o}C, under dark conditions, for 6 weeks. The epicotyl portions of

etiolated seedlings were cut transversally into 0.8-1 cm segments and used in transformation experiments.

Bacterial strain, plasmid and culture conditions

Agrobacterium tumefaciens strain EHA 101/pGA482GG was maintained on a selection plate with 50 mg l^{-1} kanamycin at 4 ^{o}C. The plasmid contains the *npt*II gene under control of the *nos* promoter, for selection on kanamycin containing medium, and the gene *uidA* under control of the CaMV promoter, for the scorable marker, β-glucuronidase (GUS) (Luth and Moore, 1999). One single colony of bacteria was inoculated into liquid YEP medium (An et al., 1988) containing 50 mg l^{-1} kanamycin and 60 mg l^{-1} gentamycin for bacterial selection, and grown overnight at 28 ^{o}C on a orbital shaker at 200 rpm. Bacteria (OD_{620} 1.0) were harvested by centrifugation at 3.500 rpm for 5 min and resuspended in liquid MS basal medium containing 100 μM acetosyringone to OD_{600nm} 1.0.

Transformation methods

SAAT and vacuum infiltration treatments

For SAAT, 15 excised epicotyls were immersed in 50 ml Falcon tubes containing 10 ml of *Agrobacterium* suspension and then subjected to ultrasound at 35W delivered by an American BrandTM Ultrasonic Cleaner (American Scientific Products, Division of American Hospital Supply Cooperation, McGaw Park Illinois, USA). The treatments differed as to sonication duration (2, 5, 10, 20, and 30 s). After sonication, the explants were maintained in *Agrobacterium* suspension for a further 15 min. Excess *Agrobacterium* suspension was removed by blotting the explants on sterile filter paper surface and the 15 explants were evenly distributed in 100 x 20 mm disposable Petri plates (Falcon, Lincoln Park, NJ) containing 20 ml of cocultivation medium (MS basal medium containing 100 mg l^{-1} myo-inositol, 10 mg l^{-1} thiamine.HCl, 10 mg l^{-1} of pyridoxine, 1 mg l^{-1} of nicotinic acid, 0.4 mg l^{-1} glycine, 25 g l^{-1} sucrose, 1 mg l^{-1} 6-benzylaminopurine (BAP), and 100 μM acetosyringone). The cocultivation was carried out in a growth chamber, in the dark, at 26 ± 2 ^{o}C for 2 days. Subsequently, the explants were transferred to selection medium [MS basal medium containing 100 mg l^{-1} myo-inositol, 10 mg l^{-1}

thiamine.HCl, 10 mg l^{-1} pyridoxine, 1 mg l^{-1} nicotinic acid, 0.4 mg l^{-1} glycine, 30 g l^{-1} sucrose, 1 mg l^{-1} BAP, 300 mg l^{-1} Timentin (Smith-Kline Beecham Laboratories, Brazil), and 75 mg l^{-1} kanamycin]. The cultures were kept at 26 ± 2 ^{o}C under 16 h of cool-white fluorescent light (76 µmol m^{-2} s^{-1}) for 7 days. The frequency of transient GUS expression was then analyzed for each treatment. Controls were treated in a similar way in the absence of *A. tumefaciens*.

For the vacuum infiltration experiment, a vacuum pump at 75 in of Hg (Barnant Co., Barrington Illinois, USA) was used to place explants under vacuum for different durations (5, 10, 15, 20, and 25 min). Again, 15 explants were used for each treatment. The same methodology applied for SAAT treatments (*Agrobacterium* inoculation, cocultivation period and selection process) was also used for vacuum infiltration experiments. The frequency of transient GUS expression was analyzed 7 days after inoculation of each treatment.

The optimum sonication and vacuum infiltration times were determined as the levels that led to a perceived increase in GUS positive foci without any perceived decrease in explant viability (Meurer et al., 1998). Furthermore, epicotyl explants were submitted to sonication and vacuum infiltration individually without *Agrobacterium* and the percentage of explants forming shoots at the end of the shoot-bud forming period (45 days after cocultivation) was recorded and correlated with explant survival and morphogenic responses.

The best treatments achieved in SAAT and vacuum infiltration experiments were combined to evaluate the effect of sonication followed by vacuum infiltration in contrast to the use of these methods alone. Transient expression levels and stable transformation were recorded as described above. For stable transformation, the explants were maintained in regeneration and selection medium for 60 days.

For all treatments, 60 and 100 epicotyl explants were used to score transient GUS expression and stable integration of foreign DNA, respectively. A control treatment was also tested, consisting of explants immersed in *Agrobacterium* suspension and MS liquid medium for 15 min.

β-glucuronidase (GUS) assay

Histochemical GUS assays were performed on epicotyl explants 7 days after inoculation with *Agrobacterium* to measure *uidA* transient expression, and after 45

days to quantify stable transformation. Epicotyl explants and tiny sections of regenerated shoots were incubated in reagent mix as described by Jefferson et al. (1987) for 4 hours at 37 ^{o}C and then de-stained in 70% ethyl alcohol for 24 h.

Molecular analysis of the transformed plants by PCR and Southern Blot

Genomic DNA was isolated from leaves using DNAzol ES Kit according to the manufacturer's protocol (Molecular Research Center, Inc., Cincinnati, OH, USA). PCR analysis was performed with the extracted genomic DNA to check for the presence of transgenes in the putative transformants using primers for both *uidA* and *npt*II genes.

The pair of primers used to amplify the *uidA* coding region was 5'-CAACGAACTGAACTGGCAG-3' and 5'- CATCACCACGCTTGGGTG-3', which amplifies a 800 bp fragment. The pair of primers for *npt*II amplification was 5'-TCACTGAAGCGGGAAGGGACT-3' and 5'-CATCGCCATGGGTCACGACGA-3'), which amplifies a 300 bp fragment. DNA samples were amplified in a PTC-200 Peltier thermal cycler (MJ Research, Inc.) using GoTaq Flexi DNA polymerase (Promega) and reaction volumes of 50 µL. The master mix for the PCR contained 0.25 µM of each primer, 0.2 mM of each dNTP and 1 x GoTaq Flexi buffer, 2.5 mM $MgCl_2$, and 1.25 U GoTaq Flexi DNA polymerase (Promega). Amplification reactions were performed according to standard protocols [initial denaturation at 94 °C for 1 min, 35 amplification cycles (denaturation at 94 °C for 30 s, primer annealing at 50°C (*uidA*) or 60 °C (*npt*II) for 30 s, and elongation at 72 °C for 45 s) and final extension step of 2 min at 72°C]. PCR products were separated by gel electrophoresis on 1.2% agarose gels, stained with ethidium bromide (0.5 µg l^{-1}) and visualized under UV light.

Ten micrograms of genomic DNA isolated from leaf tissue using DNAzol ES were digested overnight at 37°C with 100 U of *Hind*III (New England Biolabs, Ipswich, MA) in 10 mM Tris-HCl pH 7.9, 50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol to cleave a unique site in pGA482GG. The DNA was subsequently separated on a 0.8% agarose gel and transferred to a positively charged nylon membrane (Roche, Indianapolis, IN). A DIG-dUTP labeled probe was prepared using the *uidA* primers described above and plasmid DNA as template with a PCR DIG probe synthesis kit (Roche). After hybridization, the bands on the membrane

were visualized using the chemiluminescent substrate CDP-Star (Roche) and X-ray film (Kodak).

Rooting and acclimatization of plants

After 45 days in culture, putatively transformed shoots (3-5 cm in height) of Swingle citrumelo were transferred to rooting medium consisting of MS medium containing 0.5 mg l^{-1} NAA. 'Pineapple' sweet orange has low rooting efficiency *in vitro*, thus to recover whole transgenic plants the emerging shoots were transferred to a semisolid MS medium with 1.0 mg l^{-1} gibberellic acid (GA_3), for 30 days for elongation. After elongation, shoots (average 1 cm) were shoot-tip grafted *in vitro* onto Carrizo citrange seedlings. Cultures were kept in growth chamber conditions for 45 days.

After 45 days, plants with a well developed root system were transferred to sterile soil and gradually exposed to the air in a growth chamber, during an additional 15 days. After this period, the material was acclimatized in laboratory conditions in a system of shelves under illumination and with a 12 hour photoperiod of light.

Statistical analysis

A completely randomized design was used and each experiment was repeated twice. The regeneration frequency and number of shoots per explant were scored after 45 days. Transient and stable transformation frequencies were determined 7 days and 60 days after transformation, respectively. Statistical analyses were carried out using analysis of variance (ANOVA), and treatment means were analyzed using the Tukey Test ($P = 0.05$), performed with the software SAEG (Sistema de Análises Estatísticas e Genéticas, Universidade Federal de Viçosa, Brazil),

RESULTS

To identify more efficient methods for *Agrobacterium* infection of citrus, we tested SAAT and vacuum infiltration of epicotyl explants. These methods have the potential to increase gene transfer efficiency by improving penetration of *Agrobacterium* cells into the cell layers beneath the epicotyl epidermis. The control experiment (with no *Agrobacterium* added) was designed to determine whether these techniques could be used without a negative effect on epicotyl-based plant morphogenesis. Response of explants after our typical method of inoculation ('dipping' 10 min) did not differ from the nontreated control. When vacuum conditions were imposed on both cultivars tested, there was no significant decrease in the number of shoots produced per explant (Table 1). But at the longer periods of treatment, it was observed a decreased number of explants forming shoots. A similar trend was observed in the SAAT experiments, with the morphogenic response decreasing as the period of treatment increased. However, we did not observe a higher mortality effect on the infiltrated tissues or target tissues subject to SAAT, but only a relatively slight decrease in the morphogenic potential.

The second experiment was designed to examine the effect of SAAT and vacuum infiltration on GUS transient expression. Interestingly, for both cultivars tested, the vacuum infiltration treatment greatly enhanced the levels of transient expression (Table 1). Treatments ranging from 10 min to 25 min gave the highest transient expression although no significant differences were observed in the number of explants expressing GUS at 10 min and 25 min of vacuum duration.

Table 1 – Effect of duration of sonication and vacuum infiltration on explant viability and transient expression of *uidA* gene in epicotyl explants of 'Pineapple' sweet orange and 'Swingle' citrumelo

Treatments	No *Agrobacterium*					With *Agrobacterium* infection		
	No. of explants analysed	Explants producing shoots (%)		Mean number of shoots per explant		No. of explants assayed	Transient GUS+ expression of explants (%)	
		'Pineapple' sweet orange	'Swingle' citrumelo	'Pineapple' sweet orange	'Swingle' citrumelo		'Pineapple' sweet orange	'Swingle' citrumelo
Control 1	60	95.66^{a}	100.00^{a}	2.01^{a}	12.45^{a}	50	0^{e}	0^{f}
Dipping 10 min	60	92.17^{a}	98.12^{a}	1.96^{a}	11.26^{a}	101	28.69^{d}	37.66^{e}
SAAT 2 s	60	84.75^{ab}	91.66^{ab}	1.84^{ab}	9.41^{b}	100	37.31^{bcd}	58.79^{abcd}
SAAT 5 s	60	78.80^{abc}	91.92^{ab}	1.37^{bc}	9.06^{b}	106	25.44^{d}	47.13^{cd}
SAAT 10 s	60	71.66^{bc}	85.38^{abc}	1.33^{bcd}	8.03^{bc}	112	20.53^{d}	44.91^{cd}
SAAT 20 s	60	69.04^{bc}	84.41^{abc}	1.05^{cd}	5.54^{de}	102	23.08^{d}	45.64^{cd}
SAAT 30 s	60	69.51^{bc}	71.95^{bc}	1.00^{cd}	4.40^{efg}	107	21.08^{d}	48.44^{bcd}
Vacuum 5 min	60	83.80^{ab}	91.53^{ab}	1.39^{bc}	8.54^{b}	102	32.40^{cd}	46.37^{cd}
Vacuum 10 min	60	73.14^{abc}	95.23^{a}	1.30^{bcd}	6.28^{cd}	95	47.68^{abc}	66.61^{abc}
Vacuum 15 min	60	73.94^{abc}	87.04^{abc}	1.09^{cd}	5.33^{def}	104	52.00^{ab}	64.86^{abc}
Vacuum 20 min	60	67.59^{bc}	83.33^{abc}	1.04^{cd}	3.56^{fg}	99	65.12^{a}	70.93^{ab}
Vacuum 25 min	60	62.76^{c}	74.99^{bc}	0.78^{d}	3.01^{fg}	101	65.05^{a}	71.84^{a}

Means followed by the same letter, within the same column, do not differ significantly by Tukey test ($p = 0.05$).

Although target explants responded to a wide range of SAAT durations, the explants demonstrated more sensitivity to longer duration periods, displaying decreases in morphogenic responses and GUS transient expression as the period of treatment increased (Table 1). SAAT of 5 s duration resulted in fewer number of explants producing blue spots in both citrus cultivars. Therefore, 10 min of vacuum infiltration and 2 s of SAAT were selected for further experiments to investigate the combined effect of sonication and vacuum infiltration in *Agrobacterium*-mediated transformation of citrus.

Transient and stable transformation frequencies in both cultivars combining SAAT and vacuum infiltration were compared with the two methodologies alone and with the widely used 'dipping' method (Figure 1). The use of SAAT for 2 s followed by 10 min of vacuum infiltration had a positive effect on stable transformation efficiency in 'Pineapple' sweet orange, resulting in the highest stable transformation efficiency obtained (8.4%). Transformation rates were also successful using either 2 s of sonication or 10 min of vacuum infiltration alone, as compared with the standard protocol ('dipping'). The latter showed the lowest stable transformation efficiency (3.6%). For Swingle citrumelo, stable transformation efficiency was also enhanced with the combination of SAAT and vacuum infiltration (9.6%) compared to both treatments alone. But in this cultivar, even though a high level of transient GUS expression was obtained with the combined treatment, the highest frequency of stable transformation was reached with the standard protocol (11.2%).

Our results indicated that a high transient expression frequency did not necessarily result in high stable transformation frequencies. These low conversion rates of transient-to-stable transformation could be due to low efficiency of T-DNA integration. The detection of GUS expression in epicotyl explants 7 days after inoculation with *Agrobacterium* EHA 101/pGA482GG, further confirmed by stable transformation, indicated that the combination of sonication and vacuum infiltration is an efficient methodology to enhance agroinfection in citrus species.

To confirm the presence of both genes (*npt*II and *uidA*), genomic DNA of the putative transgenic and control plants was isolated. A number of putative transformants were assayed by PCR amplification of genomic DNA using a set of specific primers for both *uidA* or *npt*II genes. PCR analysis resulted in the expected sizes for the *npt*II (300 pb; Figures 2A and 2B) and *uidA* (800 pb; Figures 2B and 2D) amplified fragments. No amplified product was detected in the samples containing DNA isolated from an untransformed control plants.

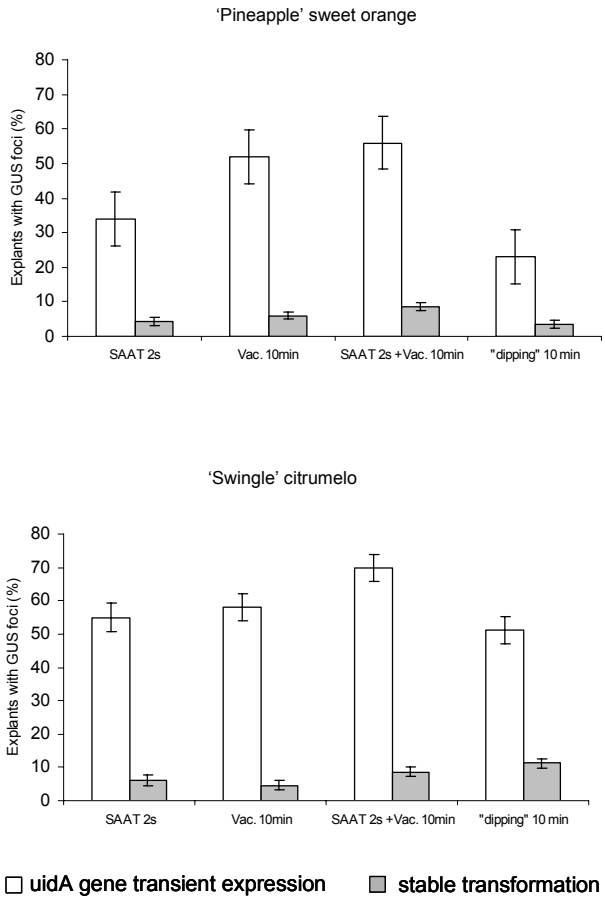

Figure 1 – Influence of SAAT (Sonication-assisted *Agrobacterium*-mediated transformation), vacuum infiltration-assisted *Agrobacterium*-mediated transformation (Vac) and standard method ('dipping') on transient expression and stable transformation of *uidA* gene in epicotyl explants of Pineapple sweet orange and Swingle citrumelo. Vertical bars indicate standard error (S.E). Stable transformation efficiency was calculated as the number of GUS + shoots divided by the number of explants inoculated in *Agrobacterium* cocultivation media.

Integration of binary vector pGA482GG into genomic DNA of Swingle citrumelo was molecularly confirmed by Southern hybridization. A Southern blot of genomic DNA from five independent events digested with *Hind*III and probed with *uidA* gene is shown in figure 2E. In this panel, three (1, 4, and 10) of the five sampled events proved to be stable transformants. The events did not present complex patterns of transgene integration in the citrus genome, containing one to three hybridization signals. This low copy of introduced DNA in transgenic tissue is typical of *Agrobacterium*-mediated transformation.

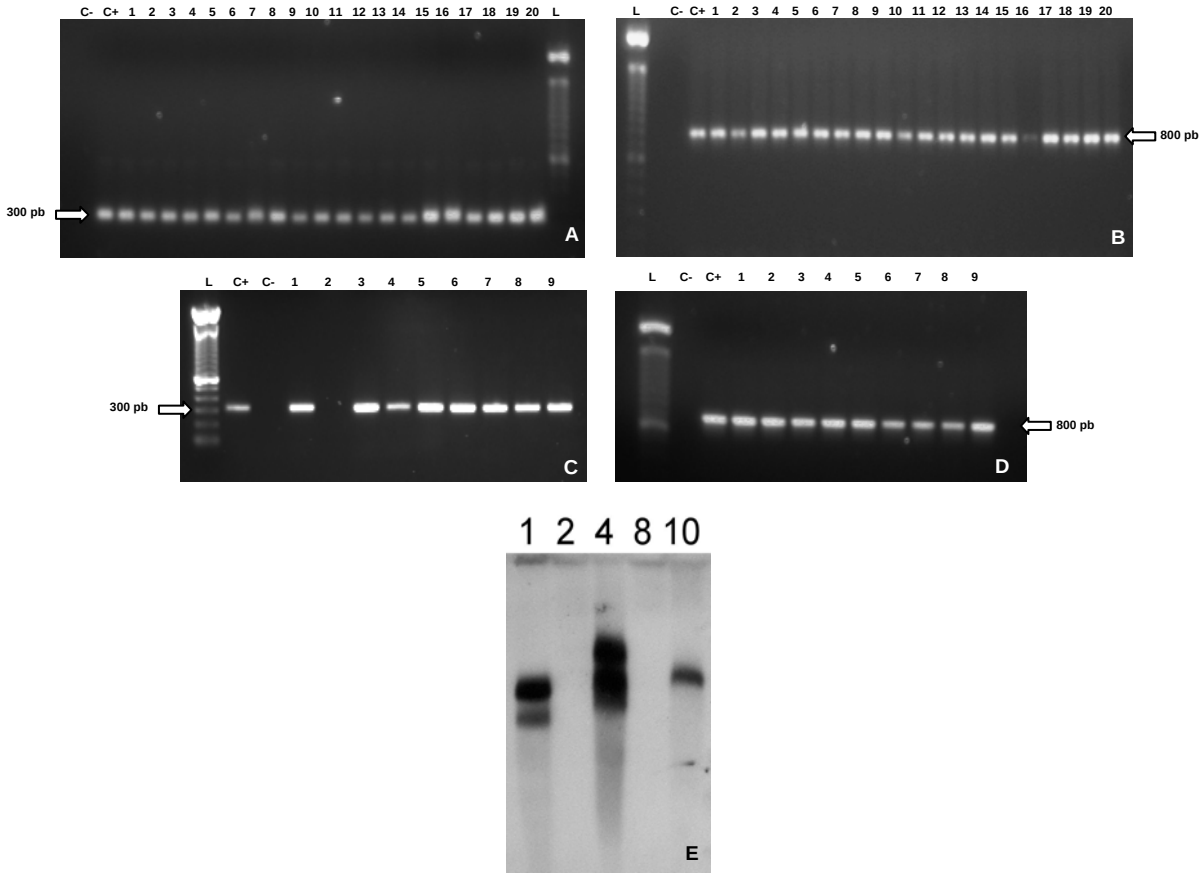

Figure 2 – PCR detection of the npt*II* and *uidA* genes in putatively transformed shoots of 'Swingle' citrumelo (A-B) and 'Pineapple' sweet orange (C-D), and Southern-blot analysis from genomic DNA from five 'Swingle' regenerants (E). **A**. PCR detection for *npt*II gene (300 bp) for Swingle citrumelo. **B**. PCR detection for *uidA* gene (800 bp) for Swingle citrumelo. **C**. PCR detection for *npt*II gene (300 bp) for 'Pineapple' sweet orange. **D**. PCR detection for *uidA* gene (800 bp) for 'Pineapple' sweet orange. For lanes 1-20 (A-B) and 1-9 (C-D) correspond to independent transgenic events. C^+ positive control (plasmid vector of transformation); C^- non-transgenic plant. L = 100 bp DNA ladder. **E**. Southern-blot analysis from genomic DNA from five 'Swingle' regenerants (lanes 1, 2, 4, 8, 10). Ten micrograms of genomic DNA extracted from leaf tissue using DNAzol ES were digested overnight at 37°C with 100 U of *Hind*III to cleave a unique site in pGA482GG. Note that DNA samples 2 and 8 did not hybridize with the *gus* probe.

DISCUSSION

Citrus has been easily transformed by means of *Agrobacterium*-mediated genetic transformation, but little is known about the use of vacuum and SAAT during agroinfection in this genus. The low transformation efficiency is a major obstacle to citrus genetic transformation. In literature, reports involving *Agrobacterium*-mediated transformation of Swingle citrumelo and 'Pineapple' sweet orange obtained 8.6% (Molinari et al., 2003) and 7.9% (Peña et al., 1995a) of transformation efficiency, respectively. Therefore, the transformation frequencies obtained in our study, 11.2% for Swingle citrumelo and 8.4 % for 'Pineapple' sweet orange, are the highest transformation frequencies reported until now.

Our results indicated that 2 s of SAAT followed by 10 min of vacuum infiltration increased frequency of transient GUS expression and stable transformation in *Agrobacterium*-mediated transformation of 'Pineapple' sweet orange. Similar results were obtained by Liu et al. (2005), who described an efficient method for the transformation of kidney bean with *lea* gene using a combination of sonication and vacuum infiltration-assisted *Agrobacterium*-mediated transformation. Among 18 combinations of transformation methods, 5 min sonication combined with 5 min vacuum infiltration enabled the highest transformation efficiency. For Swingle citrumelo, the highest frequency of stable transformation was obtained using the standard protocol, although the transient GUS expression enhanced with the use of SAAT and vacuum as compared to "dipping". The enhancement of transient GUS

expression by SAAT treatment was genotype specific, with significant enhancement in 'Swingle' explants but not in 'Pineapple'. It has been suggested that different responses among cultivars could be caused by differential response of the genotypes to wounding stress (Wordragen and Dons, 1992). However, 'Pineapple' is much more recalcitrant to *in vitro* regeneration as compared to 'Swingle', as shown by regeneration in control treatments (Table 1), and this may also have affected the results.

Several plant transformation protocols have been established using vacuum infiltration as a technique to facilitate *Agrobacterium* infection (Cheng et al., 1997; Clough and Bent, 1998; Amoah et al., 2001; Charity et al., 2002; Leelavathi et al., 2004; Ikram-Ul-Hal, 2004; Acereto-Escoffié et al., 2005; Liu et al., 2005; Canche-Moo et al., 2006). However, SAAT is currently the most important use of ultrasound in plant tissue culture (Gaba et al., 2006). A sonication treatment can stimulate shoot regeneration (see review by Gaba et al., 2006; Ananthakrishnan et al., 2007; Beranová et al., 2008), and increases both transient expression and stable transformation of several species, such as soybean (Trick and Finer, 1997; Meurer et al., 1998; Santarém et al., 1998; Trick and Finer, 2000), black locust (Zaragozá et al., 2004), *Chenopodium rubrum* (Flores Solís et al., 2007), squash (Ananthakrishnan et al., 2007), chickpeas (Pathak and Hamzab, 2008) and flax (Beranová et al., 2008). However, SAAT does not always produce positive results. For instance, SAAT was used in attempts to transform precultured wheat inflorescence tissue, and although the number of explants showing transient GUS expression doubled with a brief sonication treatment, the number of expressing areas per explant was reduced, leaving no great benefit (Amoah et al., 2001). Transient gene expression in *Pinus pinea* was greatly increased by SAAT, but the cotyledonary explants were able to survive SAAT and generate transgenic buds only at very low *Agrobacterium* concentrations (Humara et al., 1999). On the other hand, brief sonication enhanced *Agrobacterium*-mediated transient and stable transformation of *Pinus taeda,* which was further improved by the use of *Agrobacterium* containing additional virulence genes (Tang, 2003).

Sonication and vacuum infiltration can enhance the infiltration process. Sonication may create microwounding released from the cavitation of microbubbles causing minute visible wounds within and on the tissue (Gaba et al., 2006). Such phenomena might allow better access and infection of plant cells by *Agrobacterium*

(Beranová et al., 2008). In addition, the wounded tissue often produces inducers of the T-DNA transfer process, due to secretion of more phenolic compounds, enhancing the accessibility of putative cell wall binding factor to the *Agrobacterium* during transformation (Stachel et al., 1985). The use of vacuum infiltration after sonication may provide additional entry sites for bacteria, allowing the transformation of cells deeper into the plant tissue layer compared to the cells from surface that are accessible by cocultivation.

Interestingly, two of the five plants assayed in Southern blot were negative for the presence of the *uidA* gene hybridization signal. Some reasons that may have accounted for negative hybridizaton signal in two (2 and 8) of the previous gus^+ plants is the chimeric nature of the regenerants. A similar result was found by Charity et al. (2002), who showed that while putative transgenic from cotyledon explants gave positive PCR results, the same was not true by Southern analysis. Therefore, the alignment of PCR and Southern results is not stringent, and reliance on PCR alone may lead to the false identification of putative transgenic.

In conclusion, we have developed an efficient protocol for transformation of epicotyl segments of 'Pineapple' sweet orange using sonication and vacuum infiltration during *Agrobacterium* inoculation, which was demonstrated to be a promising methodology for the genetic transformation of recalcitrant citrus species.

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CAPÍTULO III

AGROBACTERIUM-MEDIATED TRANSFORMATION AND REGENERATION OF CITRUS FROM IMMATURE COTYLEDONS

ABSTRACT

In citrus, epicotyl and internodal stem segments provide the predominantly used explants for regeneration of transgenic plants following the genetic transformation experiments. The procedures involved are demanding, laborious, and time consuming and the success depend on citrus genotype. We have developed a novel and alternative shoot regeneration and transformation system for citrus that uses immature cotyledons. Using immature cotyledons of 'Duncan' grapefruit (*Citrus paradisi* Macf.), we have optimized the composition of culture medium, conditions of incubation, and the different parameters affecting T-DNA transfer. The optimized conditions were successfully used to generate stably transformed citrus plants. These results demonstrate that immature cotyledons are amenable explants to *Agrobacterium*-mediated genetic transformation and regeneration of transgenic citrus plants.

INTRODUCTION

Several protocols for regeneration of transgenic citrus plants have been reported in the past few decades. These protocols have relied on somatic embryogenesis from nucellar calli (Hidaka et al., 1990; Duan et al., 2007) or from protoplast-derived cultures (Kobayashi and Uchimiya, 1989; Vardi et al., 1990), or shoot organogenesis from epicotyl or internodal stem segments (Moore et al., 1992; Kaneyoshi et al., 1994; Peña et al., 1995a,b, 1997; Gutiérrez-E et al., 1997; Bond and Roose, 1998; Cervera et al., 1998a; Luth and Moore, 1999; Dominguez et al., 2000; Ghorbel et al., 2000; Yang et al., 2000; Costa et al., 2002; Molinari et al., 2003; Kayim and Koc, 2005). However, some citrus species of major importance, including Clementine (*Citrus clementina* Hort. ex Tan.), Satsuma (*C. unshiu* Mak. Marc.), and Cleopatra (*C. reshni* Hort. ex. Tan.) mandarins and certain sweet orange (*C. sinensis* L. Osb.) varieties, remain recalcitrant to transformation. Recalcitrance is mainly due to difficulties to regenerate shoots or somatic embryos from the transformed cells.

The amenability of the citrus species to genetic transformation could be improved if alternative regeneration-competent tissues are recognized and used as explant source in transformation experiments. An ideal tissue culture system for plant transformation must provide a large number of regenerable cells accessible to the gene transfer treatment, that will retain the capacity for regeneration for the duration of the necessary target preparation, cell proliferation, and selection treatments (Birch, 1997). Although there seems to be no reason to prefer shoot

organogenesis or somatic embryogenesis regenerative pathways, the former is advantageous since it allows, in most cases, gene transfer into intact, readily available tissue explants and regeneration with a minimal time in tissue culture. Besides epicotyl and internodal stem segments, shoot organogenesis in citrus has been also achieved from other explant sources, including shoot meristems of seedling and mature trees (Barlass and Skene, 1982), leaf sections (Chaturvedi and Mitra, 1974), root tissues (Sauton et al., 1982; Edriss and Burger, 1984; Bhat et al., 1992), and hypocotyl segments (Maggon and Singh, 1995).

In order to find another convenient and efficient explant source for shoot organogenesis as alternative to conventionally adopted epicotyl and internodal stem segments, we have evaluated the use of immature cotyledons on genetic transformation of citrus. An efficient protocol to generate transgenic citrus plants from immature cotyledonary explants depends on the establishment of a reliable and efficient regeneration and transformation system, in which different parameters need to be evaluated, including culture medium composition, incubation conditions, and positional effects of the cultured explants.

In the present report, we describe an alternative and reliable protocol for *Agrobacterium*-mediated transformation and shoot regeneration of citrus from immature cotyledons after evaluation of the effects of different parameters on the organogenesis and transformation efficiencies. These included different combinations of 6-benzylaminopurine (BAP), α-naphthalene acetic acid (NAA), indole acetic acid (IAA) and and 6-furfuryl-aminopurine (KIN) in the culture medium, pre-culture, *Agrobacterium* concentration, co-cultivation period, presence of acetosyringone in co-cultivation medium, and different methods to facilitate bacterial delivery to the target cell using ultrasound and/or vacuum pump. To our knowledge, this is the first time that immature cotyledons has been used as system for regeneration of transgenic citrus plants through shoot organogenesis pathway.

MATERIALS AND METHODS

Plant material and adventitious shoot induction conditions

Grapefruit [*Citrus paradisi* (Macf.) cv. Duncan] fruits were harvested from an open pollinated orchard at University of Florida Citrus Research and Education Center at Lake Alfred, and stored in a refrigerator at 4 ^{o}C until use. In the laboratory, seeds were collected and immersed for 1 min in 70 % (v/v) ethanol, followed by immersion in a solution containing 2.5 % (v/v) commercial bleach (Bleach, USA) and 0.1 % (v/v) Tween 20, with slight agitation for 15 min, and then rinsed three consecutive times with sterilized distilled water. The sterilized seeds were dried and the two seed coats were peeled out, zygotic and/or nucellar embryos were aseptically removed and the cotyledons were utilized as source of explants.

For the shoot regeneration experiments, explants were placed on medium containing MS salts (Murashige and Skoog, 1962), 30 g l^{-1} sucrose, 100 mg l^{-1} de myo-inositol, 10 mg l^{-1} thiamine.HCl, 10 mg l^{-1} pyridoxine.HCl, 1 mg l^{-1} nicotinic acid and 0.4 mg l^{-1} glycine. This medium was supplemented with different combinations of 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA) and 6-furfurylaminopurine (KIN), using a factorial design (Table 1). The medium was solidified with 7 g l^{-1} agar (PhytoTechnology Laboratories, USA) and brought to pH 5.7 ± 0.1 before the addition of agar, and further autoclaved at 121 ^{o}C, 1.1 atm, for 25 min. The cotyledons were placed upside down in the contact with the culture medium.

To determine the influence of the light conditions associated with explant orientation during shoot induction, the explants were cultured under three light regimes (continuous light for 6 weeks, continuous darkness for 6 weeks, and incubation for 3 weeks in the dark followed by 3 weeks in 16/8 h light and dark) combined with two explant orientations (upside down – with the adaxial surface facing the medium; and upside up – with abaxial surface facing the medium).

Each treatment was performed with 40 immature cotyledons, eight explants per Petri dish (90 x 15 mm), sealed with Parafilm tape (American National Co., USA). Cultures were incubated in darkness or 16/8-h (light/dark) photoperiod, under 76 μmol m^{-2} s^{-1} light radiation provided by two fluorescent 20 W white lamps, at 26 ± 2 ^{o}C. All experiments were repeated twice.

Root induction

Elongated shoots (2-3 cm in length) differentiated from the immature cotyledons were excised and cultured under four rooting conditions: 1) MS half-strength salt medium devoid of growth regulators (1/2 MSO); 2) MS half-strength salt medium with 0.5 mg l^{-1} NAA (1/2 MSO + 0.5 NAA); 3) MS medium devoid of growth regulators (MSO); and 4) MS medium with 0.5 mg l^{-1} NAA (MS + 0.5 NAA).

Each treatment consisted of 10 replicates, with one explant per test tube (150 x 25 mm). After 60 days of culture, rooting percentage and root length were scored. The experiment was repeated twice.

***Agrobacterium*-mediated transformation**

All experiments of genetic transformation were performed using the disarmed *Agrobacterium tumefaciens* strain EHA 101 (Hood et al., 1993) containing the binary vector pGA482GG (Slightom, 1991). Inocula were prepared as described in chapter 2 by Luth and Moore (1999). To achieve high transformation rates, several parameters were tested using *uidA* gene transient expression, as follows:

(1) Pre-culture with mannitol: pre-culture before bacteria inoculation was carried out in various mannitol concentrations (0, 0.2, 0.4, 0.6, 0.8, and 1.0 M) during 4 hours. After that, the explants were incubated for 10 min with *Agrobacterium* suspension

(OD_{600}= 0.5). Following incubation, explants were blotted dry on sterile filter paper and placed (upside down) onto semi-solid co-cultivation medium (MS basal medium containing with 2 mg l^{-1} BA, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA, 30 g l^{-1} sucrose) and incubated to growth chamber in the dark at 27 ± 1 ^{o}C for 3 days. After that, the explants were cultured on selection medium (MS basal medium containing 2 mg l^{-1} BA, 1 mg l^{-1} KIN, 1 mg l^{-1} IAA, 30 g l^{-1} sucrose, 25 mg l^{-1} meropenen and 30 mg l^{-1} kanamycin) in darkness at 27 ±1^{o}C, photoperiod 76 µmol m^{-2} s^{-1} light radiation provided by two fluorescent tubes for 7 days and the frequency of transient GUS expression was analyzed for each treatment;

(2) *Agrobacterium* concentration: cotyledonary explants were placed into 50 ml Falcon tubes (without pre-culture) with 10 ml *Agrobacterium* suspension and transferred to vacuum pump for 15 minutes. Each treatment consisted of various bacterial cell concentration (OD_{600}= 0.25, 0.50, 0.75, and 1.0). After 3 days of co-cultivation and 7 days on selection medium the frequency of transient GUS expression was analyzed for each treatment;

(3) Sonication: ten cotyledonary explants were placed in 50 ml Falcon tubes (without pre-culture) with 15 ml *Agrobacterium* suspension (OD_{600}= 0.5) and the tube was placed at the center of a bath sonicator. The treatments differed in sonication-assisted *Agrobacterium*-mediated transformation (SAAT) duration (2, 5, 10, 20, 30 s) at 35W delivered by AmericanBrandTM Ultrasonic Cleaner (American Scientific products, Division of American Hospital Supply Cooperation, McGaw Park Illinois, USA). After inoculation explants were maintained in *Agrobacterium* solution for 10 min. Excess *Agrobacterium* was blotted from the explants on filter paper, transferred to cocultivation medium for 3 days, then which transferred to the selection medium for 7 days and the frequency of transient GUS expression was analyzed for each treatment;

(4) Vacuum infiltration: tubes with 10 explants were transferred to vacuum chamber that could reach approximately 75 in. Hg with a vacuum pump (Barnant Co., Barrington Illinois, USA) and different vacuum duration (5, 10, 15, 20 and 25 min) was analyzed. The same methodology applied for SAAT treatments (*Agrobacterium* inoculation, cocultivation period and selection process) was used for vacuum infiltration experiments. Dipping procedure (explants were immersed for 15 min into

an *Agrobacterium* suspension) was analyzed to compare with sonication and vacuum infiltration procedures.

(5) Combination of SAAT and vacuum infiltration: the best treatments achieved in SAAT and vacuum infiltration were combined to evaluate the effect of sonication followed by vacuum infiltration as compared the use of these methods alone;

(6) Co-cultivation period: to determine the effects of co-cultivation period, we evaluated transient GUS expression using cotyledons inoculated with *Agrobacterium* incubated on co-cultivation medium for 0, 1, 2, 3 and 4 days;

(7) Acetosyringone: the concentrations of 0, 50, 100, and 150 mM acetosyringone (AS) were evaluated during the co-cultivation period.

Morphogenic responses of immature cotyledons to antibiotics

In order to determine the usefulness of the kanamycin resistance gene (*npt*II) for the selection of transformed citrus cotyledons, experiments were performed to determine the survival rate of isolated cotyledons explants on shoot regeneration medium [MS medium with 2 mg l^{-1} BAP, 1 mg l^{-1} KIN, 1 mg l^{-1} IAA, 3 % (w/v) sucrose and 0.7 % (w/v) agar] containing different concentrations of kanamycin (0, 15, 20, 25, 30, 40, and 50 mg l^{-1}).

The effects of the antibiotics meropenen (ABL, Brazil) and timentin (SmithKline Beecham, Brazil) were also evaluated on shoot regeneration medium (as described above) supplemented with different concentration of the antibiotics (meropenen: 0, 12.5, 25, 50, and 100 mg l^{-1}; timentin: 300 and 500 mg l^{-1}).

The plates of both experiments were placed in the darkness for 3 weeks, followed by incubation under a 16/8 h photoperiod regime, under 76 µmol m^{-2} s^{-1} light radiation, at 26 ^{0}C, for additional 3 weeks. After this period, the frequency of explants forming shoots and number of shoots per explant were recorded. Each treatment had 5 plates containing 5 explants and all experiments were repeated twice.

β- glucuronidase (GUS) assay

Histochemical GUS assays were performed on cotyledons explants 7 days after inoculation with *Agrobacterium*, in order to measure *uidA* transient expression, and 60 days after inoculation with *Agrobacterium* to quantify stable transformation. Entire cotyledons were incubated in reagent mix as described by Jefferson et al. (1987).

Molecular analysis of the transformed plants by PCR

Genomic DNA was isolated from leaves of transformed and control plants using DNAzol ES Kit, according to the manufacture's protocol (Molecular Research Center, Inc., Cincinnati, OH, USA). PCR analysis was performed with the extracted genomic DNA to check the presence of the transgene in the putative transformants using primers for both *uidA* and *npt*II genes.

The primers used to amplify *uidA* coding region were 5'-CAACGAACTGAACTGGCAG-3' and 5'- CATCACCACGCTTGGGTG-3', which amplifies an approximately 700 bp fragment. Primers for *npt*II amplification were 5'-TCACTGAAGCGGGAAGGGACT-3' and 5'- CATCGCCATGGGTCACGACGA-3', which amplifies a 300 bp fragment. DNA samples were amplified in a PTC-200 Peltier thermal cycler (MJ Research, Inc.) using GoTaq Flexi DNA polymerase (Promega) and reaction volumes of 50 µL. The master mix for the PCR contained 0.25 µM of each oligo of the external forward and common reverse PCR primers of the chosen markers, 0.2 mM of each dNTP, and 1 x GoTaq Flexi buffer, 2.5 mM of $MgCl_2$, and 1.25 U of GoTaq Flexi DNA polymerase (Promega). Standard amplification reactions were performed according to standard protocols (initial denaturalization at 94 °C for 1 min, 35 amplification cycles (denaturalization at 94 °C for 30 s, primer annealing at 50 °C (*uidA*) or 60 °C (*npt*II) for 30 seconds, elongation at 72 °C for 45 seconds and a final extension step of 2 min at 72 °C). PCR products were separated by gel electrophoresis on 1.0 % agarose gels, stained with ethidium bromide (0.5 ug/ml) and visualized under UV light.

Statistical analysis

The experiments involving adventitious shoot induction conditions, rooting and antibiotics were arranged in a completely randomized design. Data were subject to ANOVA with 5% significance level. Mean values were compared by Tukey's multiple range test, with a critical value of $p = 0.05$, performed with software SAEG (Sistema de Análises Estatísticas e Genéticas, Universidade Federal de Viçosa, Brazil).

For all parameters analyzed in experiments for optimization of transformation conditions, simple means were calculated with 5 replicates containing 10 explants per treatment.

RESULTS AND DISCUSSION

Adventitious shoot formation

In the present study, the type of plant growth regulator and the choice of cytokinin and auxin combination influenced the frequency of shoot organogenesis in immature cotyledons of 'Duncan' grapefruit and the subsequent number of shoots per explants (Table 1). The two cytokinins, BAP and KIN, exerted a positive effect on the number of segments producing shoots and number shoots per explant. The number of cotyledon explants forming shoots and the number of shoots per explant increased in higher BAP concentrations, but the optimal response depended on the levels of KIN, NAA or IAA. Significantly higher regeneration frequencies and number of regenerated shoots per explant were obtained in combinations of (1) 2 mg l^{-1} BAP and 0.5 mg l^{-1} KIN, (2) 2 mg l^{-1} BAP and 1 mg l^{-1} KIN, (3) 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA, (4) 2 mg l^{-1} BAP and 1 mg l^{-1} NAA, (5) 2 mg l^{-1} BAP, 0.5 mg l^{-1} KIN, and 1 mg l^{-1} NAA, (6) 2 mg l^{-1} BAP, 1 mg l^{-1} KIN, and 0.5 mg l^{-1} IAA, and (7) 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA. Despite NAA and IAA did not cause significant differences on cotyledon morphogenesis in some treatments, the presence of auxins enhanced root formation in high cytokinin containing medium (data not shown). A combination of 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA was thereafter used in induction medium throughout genetic transformation experiments (Figure 6B).

Table 1 – Organogenic responses of 'Duncan' immature cotyledonary explants to different concentrations of 6-benzylaminopurine (BAP), α-naphthaleneacetic acid (NAA) and 6-benzylaminopurine (BAP), indole-3-acetic acid (IAA), 6-furfurylaminopurine (KIN) in the culture medium

Plant growth regulator concentration (mg l^{-1}) **BAP x KIN x NAA**	Mean no. shoots/explant	Explants forming shoots (%)	Plant growth regulator concentration (mg l^{-1}) **BAP x KIN x IAA**	Mean no. shoots/explant	Explants forming shoots (%)
0.0 x 0.0 x 0.0	00.00^{d}	00.00^{d}	**0.0 x 0.0 x 0.0**	00.00^{d}	00.00^{d}
1.0 x 0.0 x 0.0	1.20^{cd}	52.00^{c}	**1.0 x 0.0 x 0.0**	1.20^{cd}	52.00^{c}
1.0 x 0.0 x 0.5	1.00^{cd}	44.00^{c}	**1.0 x 0.0 x 0.5**	2.60^{c}	76.00^{b}
1.0 x 0.0 x 1.0	2.72^{bc}	64.00^{b}	**1.0 x 0.0 x 1.0**	2.12^{c}	68.00^{bc}
1.0 x 0.5 x 0.0	3.68^{b}	56.00^{bc}	**1.0 x 0.5 x 0.0**	3.68^{b}	56.00^{c}
1.0 x 0.5 x 0.5	1.76^{c}	64.00^{b}	**1.0 x 0.5 x 0.5**	2.20^{c}	76.00^{b}
1.0 x 0.5 x 1.0	1.44^{cd}	56.00^{bc}	**1.0 x 0.5 x 1.0**	2.72^{c}	76.00^{b}
1.0 x 1.0 x 0.0	2.80^{b}	68.00^{b}	**1.0 x 1.0 x 0.0**	2.80^{c}	68.00^{bc}
1.0 x 1.0 x 0.5	3.84^{ab}	80.00^{a}	**1.0 x 1.0 x 0.5**	2.68^{c}	64.00^{bc}
1.0 x 1.0 x 1.0	2.52^{bc}	96.00^{a}	**1.0 x 1.0 x 1.0**	1.92^{c}	56.00^{c}
2.0 x 0.0 x 0.0	3.56^{b}	56.00^{bc}	**2.0 x 0.0 x 0.0**	3.56^{b}	56.00^{c}
2.0 x 0.0 x 0.5	4.28^{a}	92.00a	**2.0 x 0.0 x 0.5**	3.20^{b}	76.00^{b}
2.0 x 0.0 x 1.0	5.44^{a}	88.00^{a}	**2.0 x 0.0 x 1.0**	3.68^{b}	64.00^{bc}
2.0 x 0.5 x 0.0	5.28^{a}	80.00^{a}	**2.0 x 0.5 x 0.0**	5.28^{a}	80.00^{a}
2.0 x 0.5 x 0.5	2.24^{bc}	64.00^{b}	**2.0 x 0.5 x 0.5**	3.60^{b}	48.00^{c}
2.0 x 0.5 x 1.0	4.12^{a}	80.00^{a}	**2.0 x 0.5 x 1.0**	4.80^{ab}	84.00^{a}
2.0 x 1.0 x 0.0	5.12^{a}	96.00^{a}	**2.0 x 1.0 x 0.0**	5.12^{a}	96.00^{a}
2.0 x 1.0 x 0.5	2.64^{bc}	76.00^{a}	**2.0 x 1.0 x 0.5**	5.08^{a}	84.00^{a}
2.0 x 1.0 x 1.0	4.56^{a}	80.00^{a}	**2.0 x 1.0 x 1.0**	5.80^{a}	96.00^{a}

Means followed by the same letter within a column do not differ significantly by Tukey test (p = 0.05).

It has been previously reported that citrus genotypes differ in hormone requirements for shoot organogenesis from epicotyl segments (Durán-Vila et al., 1992; Ghorbel et al., 1998; Bordón et al., 2000; Moreira-Dias et al., 2001). In these studies, addition of the cytokinin BAP was found to be an essential component for shoot formation, irrespective of the system of regeneration. Our data also confirm the BAP requirement for efficient shoot organogenesis in immature cotyledons of citrus.

Effect of light regime and explant orientation on shoot regeneration

Different light conditions during the shoot regeneration process affected the efficiency of shoot regeneration in immature cotyledons of grapefruit 'Duncan' (Figure 1). An almost threefold increase in the number of shoots per explant was obtained when explants were cultivated in the dark regime for 3 weeks and then transferred for light with appearance of green adventitious bud in 2 days after light regime (Figure 6A). A longer etiolation period (6 weeks) affected negatively the regenerative potential, with significant reduction on the number of shoots per explant and regeneration frequencies of cotyledonary explants (Figure 2B). This condition also increase significantly callus induction, leading to an indirect regeneration pathway. The lowest frequency of shoot regeneration and number of shoots per explants were obtained when explants were cultured under light condition (Figure 2C).

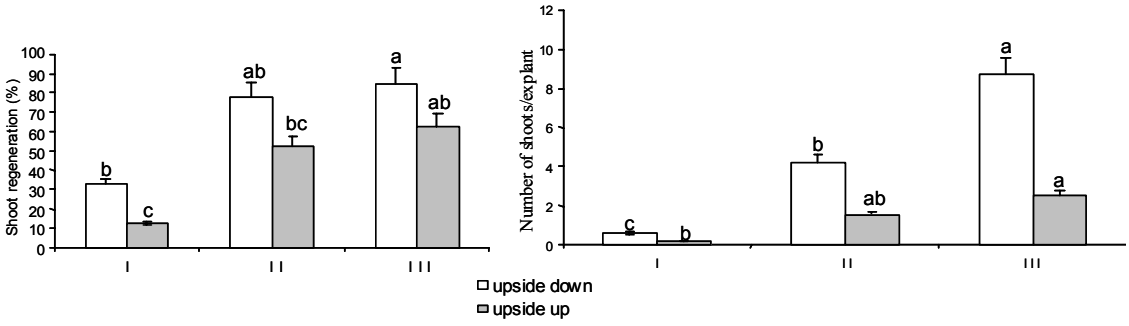

Figure 1 – Percentage of explants producing shoots and number of shoots per explant from immature cotyledons of grapefruit 'Duncan', as affected by explant orientation and light regime. **I**. explants cultured for 6 weeks in 16/8-h (light/dark) regime; **II**. explants cultured for 6 weeks in the darkness; **III**. explants cultured for 3 weeks in the dark, followed by 3 additional weeks in 16/8-h (light/dark) regime. Means followed by the same letter within the same orientation and different incubation conditions, do not differ significantly by Tukey test (p = 0.05).

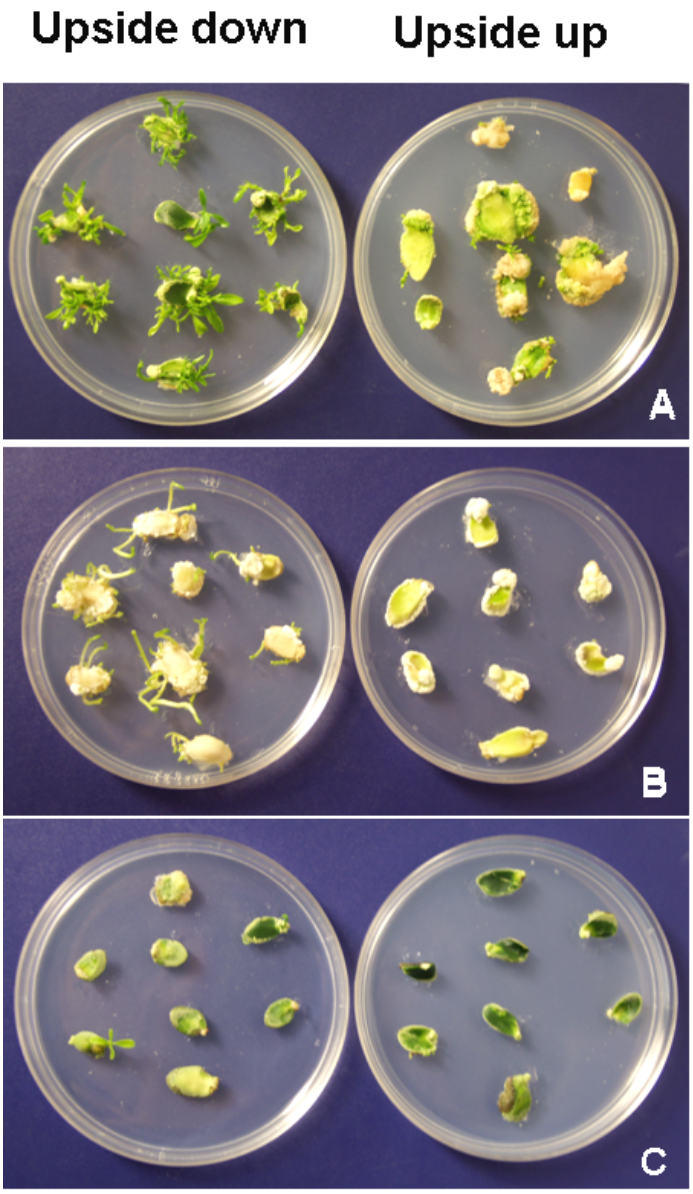

Figure 2 – Morphogenic responses of immature cotyledonary explants of 'Duncan' grapefruit (*Citrus paradisi* Macf.) as affected by explant orientation and light regime. Immature cotyledons were cultured on MS medium containing 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA. **A**. explants cultured 3 weeks in the dark followed by 3 additional weeks in 16/8-h (light/dark) regime; **B**. explants cultured for 6 weeks in the dark; and, **C**. explants cultured for 6 weeks in 16/8-h (light/dark) regime.

Orientation of the immature cotyledon explants on the culture medium proved to be another important factor affecting morphogenic responses in *citrus* (Figures 1, 2). Shoot regeneration increased significantly when the cotyledon explants were cultured upside down on the culture medium. Combination of this explant orientation with initial incubation in darkness, for 3 weeks, followed by light conditions for additional 3 weeks rendered the highest regeneration efficiencies.

Durán-Vila et al. (1992) and Pérez-Molphe-Balch and Ochoa-Alejo (1998) observed that the initial dark condition during epicotyl incubation improved considerably the bud/shoot formation. In contrast, Moreira-Dias et al. (2001) demonstrated that shoot formation in epicotyl of Troyer citrange increased when the incubation was performed in the light as compared to dark.

Effect of ionic strength and auxin on root differentiation and growth

Rhizogenic response on elongated shoots derived from immature cotyledon explants was affected by salt concentration and presence of auxin in the rooting medium (Figure 3). The higher rooting frequencies and root lenght was observed in shoots cultured on full-strength MS salts supplemented with 0.5 mg l^{-1} NAA. The root system was more vigorous as compared to other treatments (Figura 6C). Whole plants were recovered after 6 weeks, when *in vitro*-rooted plants were transferred to soil with 90 % survival efficiency. The higher rooting frequencies obtained (average 77.50) represent an important achievement of the present work. The production of whole transgenic plants of citrus, especially sweet-orange, has been hindered by difficulty in rooting the transgenic shoots (Moore et al., 1993). In most cases, *in vitro* grafting of the shoots on vigorous rootstocks has been necessary for the recovery of transgenic plants (Peña et al., 1995a, b, 1997). However, this makes the system more complicated (Perez-Molphe-Balch and Ochoa-Alejo, 1998) and time-consuming.

The auxin NAA was effective in inducing rooting in elongated Duncan shoots. Accordingly, the auxins NAA and IBA, alone or in combination, have been demonstrated to be essential for *in vitro* rooting of citrus shoots, although the optimal concentrations of these hormones for root differentiation and growth vary according to citrus genotype (Sim et al., 1989; Moore et al., 1992; Singh et al., 1994; Moreira-Dias et al., 2001; Al-Bahrany, 2002). The regenerative cotyledon-based system may represent an important alternative to successfully root adventitious shoots of citrus species recalcitrants to *in vitro* rooting.

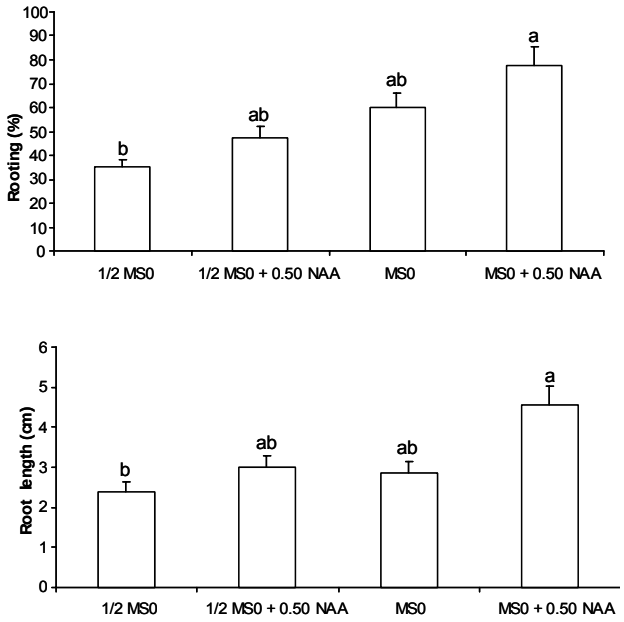

Figure 3 – Rooting responses of immature cotyledon-derived shoots of 'Duncan' grapefruit (*Citrus paradisi* Macf.) as affected by salt strength and presence of NAA. Means followed by the same letter do not differ significantly by Tukey test ($p = 0.05$).

Effects of antibiotics on shoot regeneration

Before the transformation experiments, it is important to evaluate the effects of different concentrations of the selective agents on regeneration medium in order to identify the most suitable concentration for selecting transformed shoots. Since most of the vectors for genetic transformation of plants contain the gene *npt*II from *Escherichia coli* as selective marker, immature cotyledons from 'Duncan' were incubated on basal shoot regeneration medium containing 2 mg l^{-1} BA, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA, supplemented with different concentrations of the antibiotic kanamycin. In general, it was observed that immature cotyledons were highly sensitive to kanamycin (Figure 4). kanamycin concentration at 30 mg l^{-1} showed to be detrimental to organogenic potential, without leading to necrosis and visible browning, but limiting cotyledon expansion, and reducing survival rates. At lower concentrations (10-20 mg l^{-1}), kanamycin was not effective in to restrict shoot-bud differentiation (Figure 4), as also observed in experiments of genetic transformation (Figure 6F). In concentration up to 40 mg l^{-1} kanamycin, all explants turned brown, with inhibited growth. These results suggest that 30 mg l^{-1} kanamycin would be an

effective concentration for selection of transgenic shoots in transformation experiments. Concentrations of kanamycin ranging from 50-100 mg l^{-1} are widely used for selection of transgenic shoots from epicotyl or internodal stem segments of citrus (Kaneyoshi et al., 1994; Moore et al., 1992; Gutiérrez-E. et al., 1997; Peña et al; 1995a, 1995b, 1997; Cervera et al., 1998a; Yang et al., 2000; Almeida et al., 2003), which in most cases do not prevent the development of escaped shoots. Our results indicate that immature cotyledons are more sensitive to the toxic effects of kanamycin than the conventionally adopted explants in genetic transformation of citrus, which can be considered an advantage for reduction of the commonly observed high frequency of escapes.

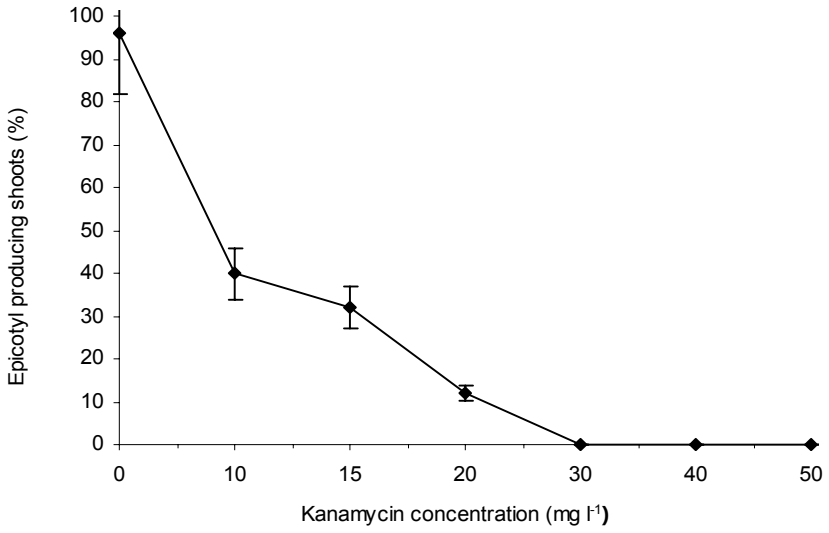

Figure 4 – Shoot regeneration from immature cotyledon explants of *Citrus paradisi* cv. Duncan, after 30 days in culture, as affected by kanamycin concentrations. Vertical bars indicate standard error (S.E).

The effects of the antibiotics timentin and meropenen, used for *Agrobacterium* control, on morphogenic responses from of the immature cotyledons were also evaluated. Interestingly, meropenen at 12.5 or 25 mg l^{-1} increase significantly the number of shoots per explant and percentages of explants forming shoots as compared to control treatment (without antibiotics) (Table 2). A similar response as to the number of shoots per explant was also observed when immature

cotyledons were incubated in culture medium containing 300 mg l^{-1} timentin. Meropenen concentrations (12.5 and 25 mg l^{-1}) and timentin at 300 mg l^{-1} enabled greater averages of number of shoots per explant. Meropenen at 12.5 mg l^{-1} was further selected for optimization of transformation conditions as described below.

Table 2 – Effect of the antibiotics timentin and meropenen on shoot formation of cotyledons explants of 'Duncan' grapefruit, after 30 days of culture

Antibiotic concentration (mg l^{-1})	Number of shoots/explant	Explants forming shoots (%)
Control	5.80[ab*]	89.00[ab]
Meropenen		
12.5	6.12[a]	92.00[a]
25	6.64[a]	90.00[a]
50	2.20[c]	56.00[d]
100	1.44[c]	48.00[d]
Timentin		
300	6.40[a]	77.00[bc]
500	5.08[b]	64.00[c]

Means followed by the same letter within a column do not differ significantly by Tukey test ($p = 0.05$).

Meropenen is a new generation carbapenen antibiotic, containing in their structure a β-lactam ring common to the penicillin and cephalosporins (Blumer, 1997). It has been recently reported that meropenen was more active against *Agrobacterium* and also improved the transformation efficiency in tobacco, tomato and rice as compared to cefotaxime and carbenicillin (Ogawa and Mii, (2004, 2005, 2007). Timentin, a penicillin derivate antibiotic associated to clavulanic acid, has been also reported to influence positively shoot organogenesis in different species (Nauerby et al., 1997; Cheng et al., 1998; Ling et al., 1998; Costa et al., 2000). Cefotaxime is the most widely used antibiotic in citrus transformation protocols (Kaneyoshi et al., 1994; Peña et al., 1995a; Cervera et al., 1998a; Cervera et al., 1998b; Gutiérrez-E et al., 1997; Bond and Roose, 1998; Pérez-Molphe Balch and Ochoa-Alejo, 1998; Luth and Moore., 1999; Domínguez et al., 2000; Yang et al.,

2000; Costa et al., 2002; Molinari et al., 2003). For transformation of immature cotyledons, timentin can be considered as the antibiotic of choice to be used to suppress bacteria growth due to four advantages: it has a positive effect on shoot regeneration, it is more economical than meropenen and cefotaxime, it is more light stable and more resistant to inactivation by β-lactamase than cefotaxime due to the presence of potassium clavulanate (Ling et al.,1998).

Optimization of transient transformation conditions

To optimize the conditions of genetic transformation using immature cotyledons as source of explants, five experiments were performed to establish the better conditions for T-DNA delivery by *Agrobacterium,* as judged by the *uidA* gene expression.

In the first set of experiment, we observed that preculture of explants with mannitol, prior to inoculation with *Agrobacterium*, remarkably reduced the frequency of GUS expression as compared to the treatments without pre-incubation or pre-incubation with MS0 devoid of mannitol (Figure 5A). Li et al. (2007) have reported a positive influence of the pre-conditioning of explants by osmoticum, such as 0.4 M mannitol. It is presumed that the recoverable plasmolysis of the cells facilitates the transfer to T-DNA from *Agrobacterium* to target cells.

In the set of experiments involving SAAT or vacuum infiltration, it was observed that transient GUS expression decreased when duration of sonication increased, while vacuum infiltration for 15 min and up increased GUS expression as compared to control (Figure 5B). However, vacuum infiltration superior to 15 min caused *Agrobacterium* overgrowth and difficulties to eliminate bacteria, resulting in the loss of explant viability. Tissue disruption, as reflected by microwoundings at cotyledon surface, was obseved after sonication treatment (Figure 6G). The combination of sonication and vacuum infiltration decreased the transient transformation frequencies as compared to vacuum infiltration alone (Figure 5C). A positive effect of vacuum infiltration during *Agrobacterium* incubation period on transformation efficiency has been reported in several species (Amoah et al., 2001; Charity et al., 2002; Ikram-Ul-Haq, 2004; Acereto-Escoffié et al., 2005; Canche-Moo et al., 2006), by improving penetration of *Agrobacterium* cells into the cell layers beneath the cotyledons epidermis. A 15 min vacuum infiltration was used in the subsequent experiments of genetic transformation.

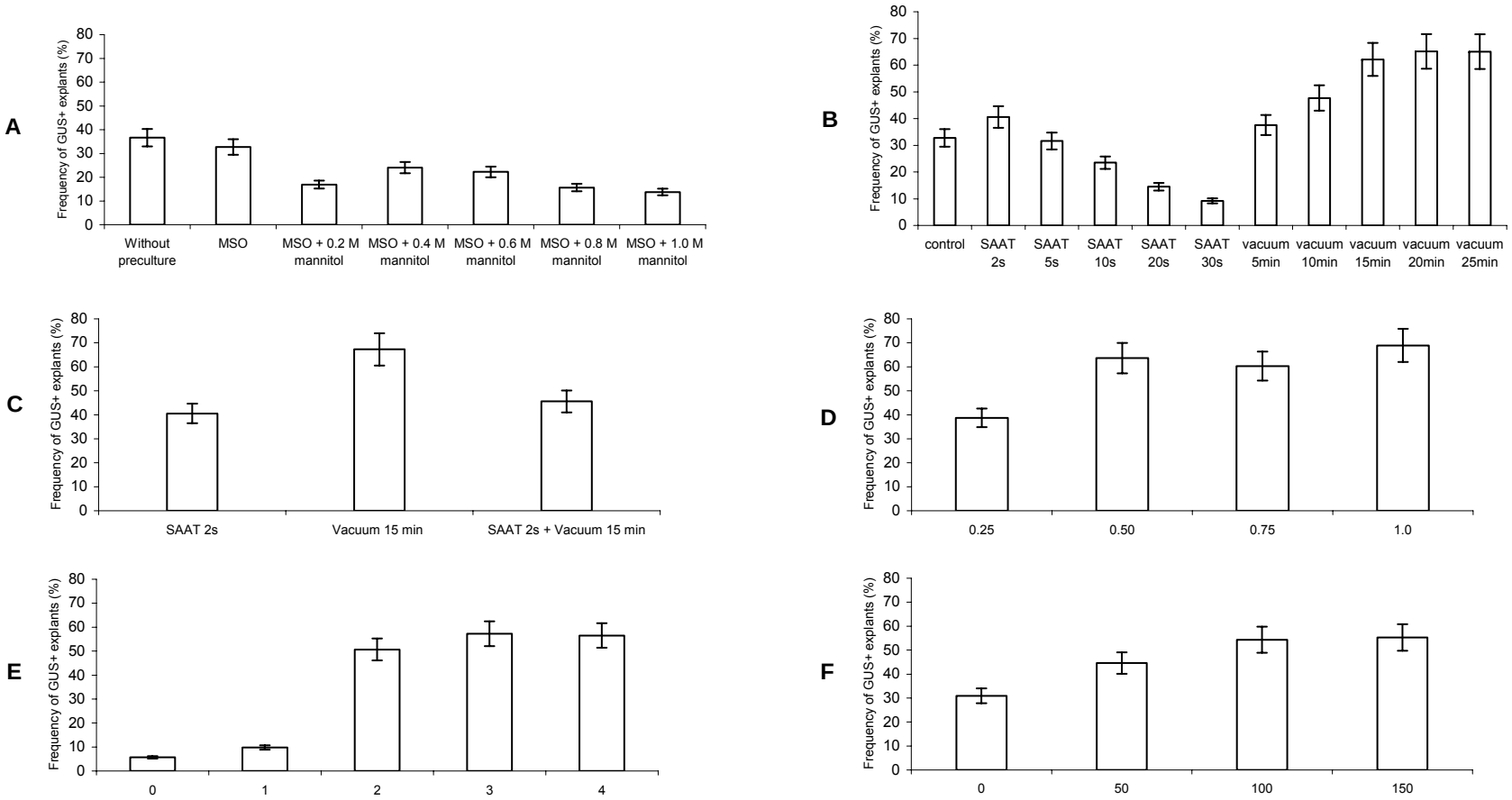

Figure 5 – Parameters affecting T-DNA delivery into immature cotyledons by *Agrobacterium.* **A**. preculture treatments; **B**. and **C**. different inoculation methods using ultrasound and/or vacuum chamber; **C**. combination of SAAT and vacuum; **D**. *Agrobacterium* concentration ($OD_{600\ nm}$); **E**. co-cultivation period (days); and, **F.** presence of acetosyringone (mM) in co-cultivation medium. Vertical bars indicate standard error (S.E).

Bacterial concentrations of 0.5 and up at OD_{600} produced similar GUS+ frequencies, which were higher than those observed at 0.25 (Figure 5D). However, bacterial density of 1.0 resulted in a severe *Agrobacterium* overgrowth, reducing the morphogenic ability of the cotyledonary explants to form adventitious buds. Bacterial concentrations of 0.50 and 0.75 were chosen to support subsequent transformation experiments.

Co-cultivation periods of 2, 3, and 4 days increased the transient GUS expression rates as compared to 0 or 1 day co-cultivation (Figure 5E). However, the co-cultivation period of 4 days resulted in remarkable *Agrobacterium* overgrowth on the explant surface, leading to explant losses. It was not observed for 3 days co-cultivation period, that was further selected to be used as routine.

Inclusion of acetosyringone (AS) in the co-cultivation medium also increased the frequencies of GUS+ explants as compared to medium without acetosyringone (Figure 6D). Increasing AS to 150 mM showed no difference as compared to concentration of 100 mM. Therefore AS was used at 100 mM in all subsequent experiments. It has been previously reported that AS has a positive effect on transformation efficiencies of epicotyl and internodal stem segments of citrus (Bond and Roose, 1998; Cervera et al., 1998a; Yang et al., 2000). AS is a phenolic compound produced during wounding of plant cells that induces the transcription of the virulence genes of *Agrobacterium* (De la Riva et al., 1998), positively favoring the interactions of agrobacteria cells and host tissues, and consequently improving transformation efficiencies.

These optimized transformation conditions were combined and used for stable genetic transformation. The transformation efficiency observed in Table 3 was 8.82 %. After 45 days, the GUS+ shoots (Figure 6E) were transferred to rooting medium to posterior PCR analyses. It revealed the presence of the inserted *uidA* and *npt*II genes fragments in the genomic DNA, resulting in the expected sizes for the *uidA* (800 pb; Figure 6H) and the *npt*II genes (300 pb; Figure 6I).

Table 3 – Transformation efficiency of 'Duncan' immature cotyledonary explants [a].

Number of explants inoculated	Number of shoots regenerated	Number of GUS + shoots/ PCR+	Transformation efficiency (%)
102	37	9/9	8.82

[a] Transformation efficiency was calculated as the number of PCR + shoots divided by the number of explants inoculated in *Agrobacterium* co-cultivation media.

Finally, we have established for the first time a simple and reliable procedure for *Agrobacterium*-mediated transformation of citrus using immature cotyledons as source of explants. This protocol can be successfully used as alternative to transformation of recalcitrant citrus species. Furthermore, this shoot regeneration system based on immature cotyledons may facilitate the effective introduction of particle-bombardment technology for transformation of citrus species that are difficult to transform with *Agrobacterium*. It has been recently reported that cotyledons can be also used to regenerate recalcitrant citrus species via somatic embryogenesis pathway (Khawale and Singh, 2005).

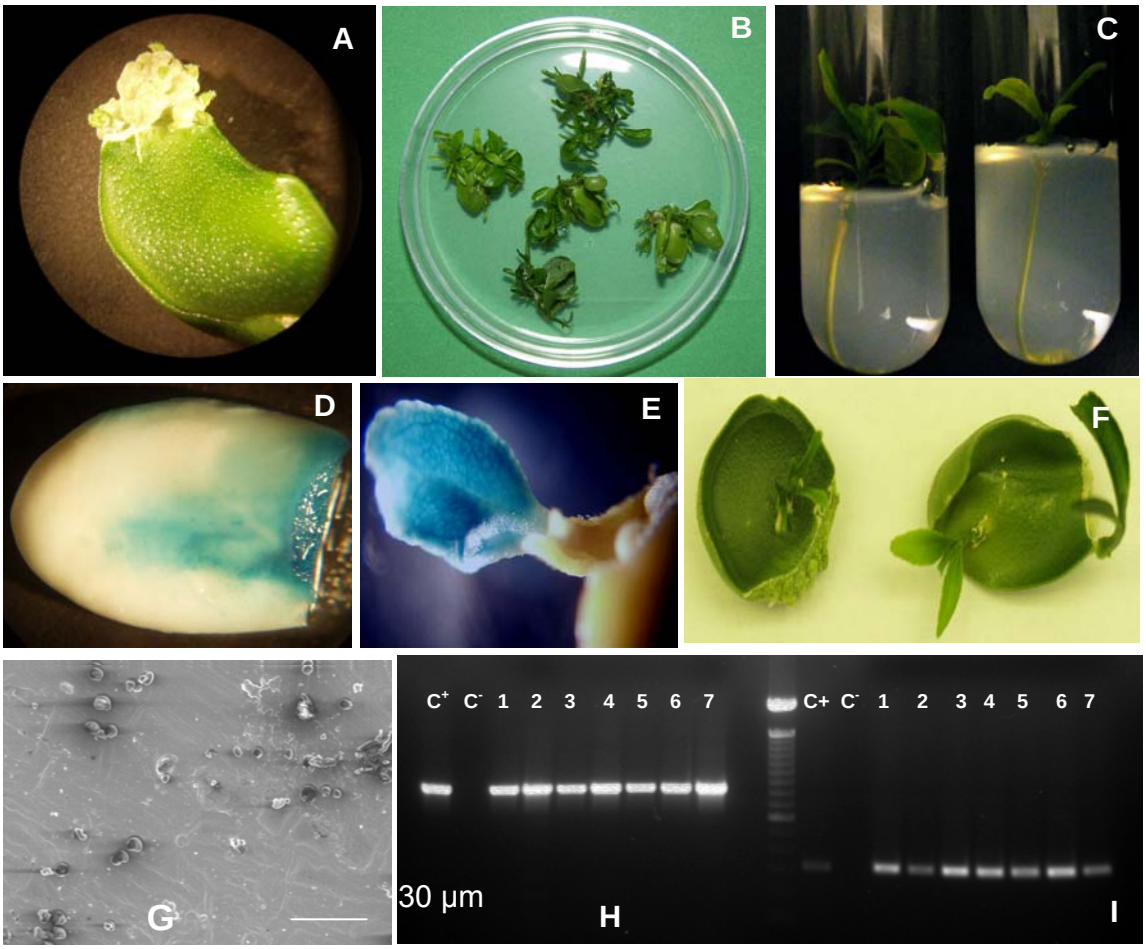

Figure 6 – *In vitro* morphogenesis and *Agrobacterium tumefaciens*-mediated transformation of 'Duncan' from immature cotyledons. **A**. adventitious bud formation on cut edge of cotyledon after 23 days of culture in MS-based medium containing 2 mg l^{-1} BAP, 1 mg l^{-1} KIN and 1 mg l^{-1} IAA; **B**. multiple adventitious shoots after 40 days in culture; **C**. rooted shoot after 30 days in MS containing NAA at 0.5 mg l^{-1}; **D**. transient GUS expression on cotyledon surface; **E**. transient GUS expression on immature cotyledons; **F**. shoot differentiated from the cotyledon surface showing resistance to kanamycin (10 mg l^{-1}), as reflected by its green color; **G**. scanning electron micrograph of cotyledon surface showing microwoundings caused by ultrasound; **H**. PCR analysis using *uidA* gene primer (800 pb); and **I**. PCR analysis using the *npt*II gene primer (300 pb).

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