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In vitro regeneration of sour orange (*Citrus aurantium* L.) via direct organogenesis

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Abstract

Low cell competency for regeneration and transformation is the main cause of so-called recalcitrance to transform a species or a genotype. A research was conducted to determine the optimum conditions for *in vitro* plant regeneration involving organogenesis in *Citrus aurantium*, which is an important rootstock worldwide. Seeds with peeled teguments were germinated *in vitro*, either kept in dark for 6 weeks or maintained in the absolute dark for 4 weeks followed by 10 days in 16-h photoperiod (56 µmol m^{-2} s^{-1}) at 27 ± 2°C. Epicotyl-originated explants were cultured in MS medium supplemented with 6-benzylaminopurine (BAP) (0, 1, 2 and 3 mg L^{-1}) and Naphthaleneacetic acid (NAA) (0, 0.05, 0.1 and 0.2 mg L^{-1}) to induce organogenesis. Effects of Pre-culture in liquid MS medium (0, 1 and 2 days) on the number of responsive explants (RE) have been also evaluated. In the next step, explants having buds were transferred to MS medium containing Gibberellic Acid (GA_3) (0, 0.5 and 1 mg L^{-1}) and the size and number of shoots, which have been produced by RE are then measured. The highest percentage of responsive explants (90%) obtained by using 2.5 mg L^{-1} BAP in combination with the 0.05 mg L^{-1} NAA which had 2 days pre-culture period of epicotyls for allowing to grow in the absolute darkness for 4 weeks, followed by 10 days in 16-h photoperiod (56 µmol m^{-2} s^{-1}). The highest number of well-developed shoots was 4.2 shoots per explant and obtained with medium containing 0.5 mg L^{-1} GA_3. These protocols are suitable in association with *Citrus aurantium Agrobacterium*-mediated genetic transformation.

Keywords: Adventitious bud; Epicotyl; Genetic transformation; Sour orange regeneration.
Abbreviations: LC_light conditions; D+L_darkness followed light; BAP_6-benzylaminopurine; GA_3_gibberellic Acid; NAA_ naphthaleneaceticacid; RE_responsive explants; KIN- kinetin.

Introduction

Improvement of *Citrus spp*. by conventional breeding methods is hampered by various aspects of *Citrus* biology such as nucellar polyembryony, heterozygosity, sexual incompatibility and long juvenile period (Grosser and Gmitter, 2005). With the recent advances of plant biotechnology, it is possible to introduce exogenous genes in the plant genome, using gene transfer techniques. However, for efficient transgenic plant production, a previously defined tissue culture system for better plant regeneration, in association with a genetic transformation system for the gene introduction are necessary (Perez-Molphe-Balch and Ochoa-Alejo, 1998). Accordingly, genetic transformation remains the main alternative in *Citrus* breeding programs such as development of *Citrus tristeza virus* (CTV) tolerant sour orange plants. The success of a genetic transformation program depends on the availability of an *in vitro* protocol that permits a high efficiency in shoot regeneration (Garcia-Luis et al., 2006).

New organs such as shoots can be induced to form on cultured explant tissues. Such freshly originated organs are said to be adventives or adventitious (George, 2008b). The creation of new forms and organization is termed morphogenesis or organogenesis. The new form, considered to be direct when bud regeneration occurs without callus, or otherwise which defined as an indirect (Phillips, 2004). So far, it has been possible to obtain the *(*adventitious) formation of shoots (caulogenesis) by the cultivation of epicotyl and intermodal segments in culture mediums containing BAP (Moura et al., 2001; Almeida et al., 2002; Silva et al., 2005) or BAP in combination with NAA (Pérez-Molphe-Balch and Ochoa Alejo, 1998, Bordon et al., 2000; Moreira-Dias et al., 2000, 2001).

Cytokinins are very effective in promoting direct or indirect shoot initiation. Many aspects of cellular differentiation and organogenesis in tissue and organ cultures have been found to be controlled by an interaction between cytokinin and auxin concentrations (Woodward and Bartel, 2005). A balance between auxin and cytokinin growth regulators is most often required for the formation of adventitious shoot and root meristems. Interactions between the two classes of regulant are often complex, and more than one combination of substances is likely to produce optimum results (Sugiyama M, 1999).

Light is often necessary for the formation of adventitious shoots from callus or explants, but this is not always the case. A short period of darkness sometimes promotes shoot morphogenesis. Adventitious buds did not form on swollen needle primordia of *Picea pungens* unless cultures were kept in the dark for 8 days. Subsequent shoot development required 16 h under 1000 lux illumination (Misson et al., 1982). The formation of adventitious buds on young flower buds of *Freesia*

was greatly improved if the explants were grown in the dark before being transferred to the light (Pierik and Steegmans, 1975).

Shoots are occasionally treated with GA_3 to increase the length of shoots during multiplication; or prior to rooting, when it is usually applied at a special elongation stage i. e. after shoot multiplication and before shoots being harvested. Shoot cultures of the salt-tolerant plant *Atriplex canescens* were found to remain stunted without the application of GA_3 (Wochok and Sluis, 1980). However, when GA_3 is added to plant tissue culture media, it often prevents direct shoot regeneration for example in *Begonia* leaf discs (Chlyah, 1972).

It is notable that many natural and unnatural stresses such as heat, cold, drought, salinity and synthetic growth regulators can initiate metabolic events similar to those caused by wounding (Bowles, 1991). It is unsurprising therefore that wounding tissue often leads to effects such as those seen after application of various substances such as hormones, elicitors and synthetic growth regulators for example effects on morphogenesis (Lo, 1997).

Also, some other factors that have major effects on shoot regeneration like, type of the explant (Bordon et al., 2000; Moreira-Dias et al., 2001; Schinor, 2011), light treatment (Almeida et al., 2002; Mendez et al., 2002; Azevedo et al., 2006; Silva et al., 2010) and pre-culture conditions (Hao, 2000; Li et al., 2003) have been evaluated. However, some important rootstock genotypes such as *C. aurantium* and *C. volkameriana*are known to have the extremely low response to the bud induction (Costa et al., 2004; Tavano et al., 2009).

In the present experiment, we evaluated *in vitro* organogenesis and direct adventitious buds formation of sour orange considering culture media, type of explant and a pre-culture condition. The protocol might be used for genetic transformation experiments of this species.

Results and Discussions

The experiment was carried out in an independent and stepwise approach. In this approach, the best combination of BAP and NAA concentrations, were considered in two different light regimes D or D+L. Subsequently, pre-culture and shoot growth tests were carried out based on the highest percentages of RE obtained from previous steps.

Effect of plant growth regulators on bud induction

Evaluation of RE was made after 5 weeks of culture. Hence the explants with adventitious bud length higher than 1mm were counted as responsive. The evaluation revealed that BAP has a significant effect on regeneration, because no regeneration was observed in the absence of BAP (Table 1). Explants in hormone-free treatments, showed the sign of swelling. However, in few cases the formation of hard callus at cutting edges with no bud regeneration was also observed. The highest percentage of RE occurred in regeneration medium, containing 2.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA (60%, Fig. 1). When BAP was used as the sole source of hormone in regeneration medium explants, regeneration happen to be more quickly (2 weeks) without any callus formation. In this type of regeneration media (lack of NAA), the highest rate of RE (53.3%) was obtained at a concentration of 2.5 mg L^{-1} (Fig. 1 and Table 1).

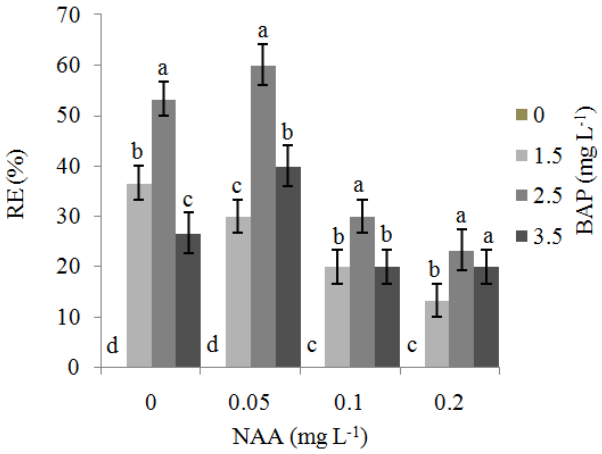

Fig 1. Effects of combinations of BAP and NAA (mg L^{-1}) on percentage of responsive explants, extracted from seedlings after grown in absolute darkness for 5 weeks.

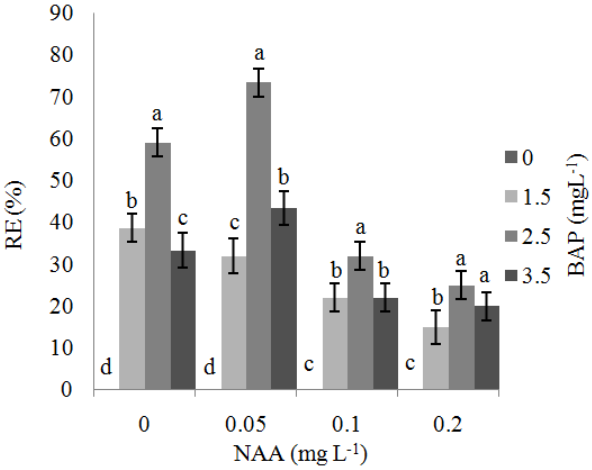

Fig 2. Effects of combinations of BAP and NAA (mg L^{-1}) on percentage of responsive explants, extracted from seedlings after grown in absolute darkness for 4 weeks followed by 10 days in 16-hour photoperiod (56 µmol m^{-2} s^{-1}).

Most often, a balance between auxin and cytokinin growth regulators is required for the formation of adventitious shoot and root meristems. The requisite concentration of each type of regulant, differs greatly according to the genotype (Almeida et al., 2002; Schinor, 2011), medium composition and plant growth related factors (i.e. explant age and type), incubation conditions, explant orientation in medium and light conditions (Bordon et al., 2000; Moreira-Dias et al., 2000; Costa et al., 2004; Silva et al., 2005). Besides, more than one combination of substances is likely to produce optimum results (Moshkov et al., 2008). It has been observed that BAP highly induces the formation of adventitious shoots, while kinetin (KIN) allows normal shoot growth. Regarding to regeneration of explants in *Citrus*, using BAP is more effective than KIN (Moreira-Dias et al., 2001; Almeida et al., 2002; Silva et al., 2005) and thidiazuron (Germana et al., 2011).

Table 1. Analysis of variance regarding the effects of different concentrations and combinations of BAP, NAA and light condition on regeneration of buds from epicotyl-originated explants of *C. aurantium*.

SOV	DF	MS	F-Value
BAP	3	41.545833	302.15**
NAA	3	8.6791667	63.12**
LC	1	7.0123377	50.99**
BAP*NAA	9	2.723611	19.81**
LC*BAP	3	2.3253678	16.91**
LC*NAA	3	1.0436782	7.59**
LC*BAP*NAA	9	0.3897657	2.83**
Error	54	0.1375000	

** Means are significantly different at p≤0.01

According to Tavano et al. (2009), the highest percentages of responsive epicotyl-derived explants (59%) achieved at 0.5 mg L^{-1} BAP and maximum number of adventitious shoots per explants (1) was obtained at the same hormone concentration. Silva et al. (2010) by culturing *C. aurantium* epicotyl segments in MT (Murashige and Tucker, 1969) medium containing 1 mg L^{-1} BAP and 0.3 mg L^{-1} NAA were able to achieve 80.9% RE from which 3.1 buds per explants attained.

Although it is well reported that *Citrus in vitro* organogenesis is favored by culture media supplemented with cytokinin, the development of adventitious shoots may occur in basal medium, especially when very young explants are used (Schinor et al., 2011; Almeida et al., 2002; Costa et al., 2004). In other words, the supplementation of culture media with the combination of BAP and NAA did not result in an increase of the percentage of responsive explants. It might be as a result of culture media composition and genotype.

The responsive rates lower than 50% are not considered as adequate for genetic transformation. High efficiency in shoot regeneration is extremely important for genetic transformation, because as it increases the chance of obtaining transgenic plants (Tavano et al., 2009). Therefore, our final 92% efficiency in regeneration, obtained in pre-culture experiment, is a promising achievement regarding *Agrobacterium*-mediated transformation.

Effect of light conditions on bud induction

Light conditions had a significant effect on bud formation and on the number of RE. Explants that were extracted from seedlings and maintained in the absolute darkness for 4 weeks followed by 10 days in 16-h photoperiod (56 µmol m^{-2} s^{-1}), had a significant higher number of RE than the explants that have been taken from seedlings and kept in the absolute darkness for 6 weeks (73.3% vs. 60%, respectively; Fig. 2 and Fig. 3).

In some studies (Azevedo et al., 2006; Silva et al., 2010), explants from *in vitro* grown seedlings that were kept in the absolute darkness for 3 weeks and subsequently for a week in 16-h photoperiod (56 µmol m^{-2} s^{-1}), were used for transformation and regeneration.

The light-induced development of structure or form (photomorphogenesis), which induces a rapid change in gene expression leads to the normal pattern of development (George and Davies, 2008) might be the main reason for a higher number of buds or RE in explants extracted from light-grown seedlings.

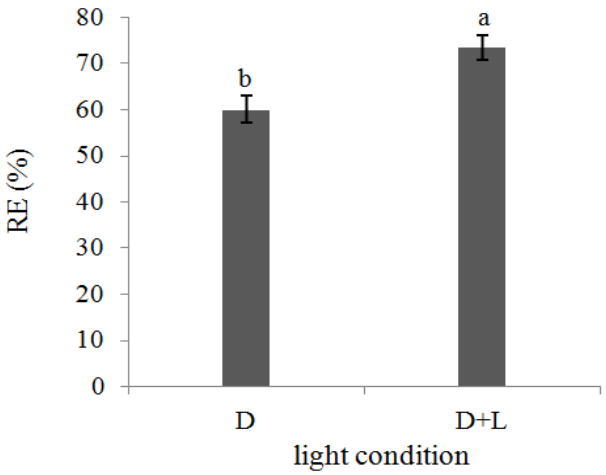

Fig 3. Effects of light conditions on percentage of responsive explants at the presence of 2.5 mg L^{-1} BAP and 0.05 mg L^{-1} NAA, D: 5 weeks Absolute darkness; D+L: 4 weeks absolute darkness followed by 16-h photoperiod (56 µmol m^{-2} s^{-1}).

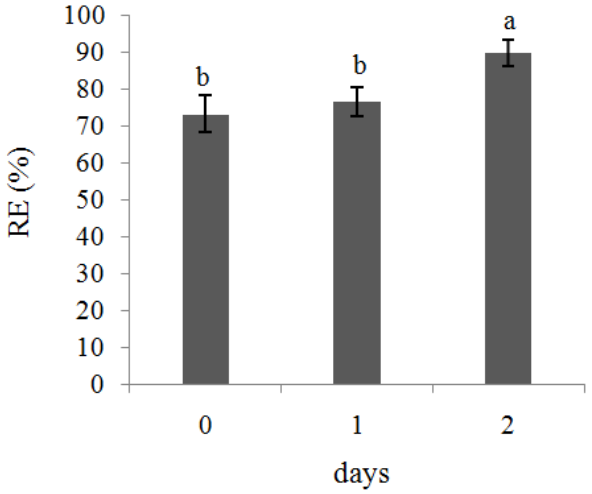

Fig 4. The effects of pre-culture length (days) on percentage of responsive explants. Explants extracted from seedlings grown at D+L condition and in the presence of 2.5 mg L^{-1} BAP plus 0.05 mg L^{-1} NAA.

Effect of pre-culture on bud induction

Evaluation of the results revealed that 2 days pre-culture using epicotyls in liquid medium had a considerable effect on shoot production (90%; Fig 7 A) and the number of RE was significantly higher than for 0 and 1 day pre-culture period (73.3% and 76.6%, respectively; Fig. 4).

Li et al. (2003) has reported that pre-cultivation of Valencia sweet orange (*Citrus sinensis*) callus for 4 days, had the best result on transformation. Suspension culture of *Citrus* embryogenic callus for 4-8 days caused the logarithmic growth phase (Hao, 2000). Explant pre-cultivation of plum tried by Mante et al. (1991) apricot by Laimer et al. (1992) and Arabidopsis by Sangwan et al. (1992) showed an increase in the number of competent cells.

Villemont et al. (1997) reported that pre-cultivation of Petunia leaf explant in medium containing 2,4-D and BAP for 2-3 days, before transformation with *Agrobacterium*, is highly enhanced genetic transformation. During pre-culture period, cells were actively dividing and duplicating DNA. Throughout a cell cycle, S-phase (DNA duplication) has demonstrated to be absolutely necessary for *Agrobacterium*-mediated transformation or T-DNA transfer (Pena et al., 2004).

In addition, advantages of liquid media for the culture of organs may be due to a greater surface area which the explant might be in contact with a liquid, as well as the reduction of diffusion gradients for nutrients and gases between medium and explant. The combination of these two factors, allows more efficient uptake of nutrients and growth regulators, and the partial increase in pressure of oxygen in tissues. There may also be direct effects of low oxygen tensions on membrane functioning and on water and ion uptake capacity (Jackson et al., 2003).

Effect of GA_3 on shoot regeneration/ elongation of adventitious shoots

Five weeks after keeping in regeneration medium, explants with a 5 mm long shoot were transferred into shoot regeneration medium (Fig 7 B). The highest number of shoots per explant was obtained from 0.5 mg L^{-1} GA_3 treatments (4.2), which was significantly higher than treatments with 0 and 1 mg $L^{-1}GA_3$ (2.68 and 3.36, respectively; Fig. 5).

In our experiment, explants in 0.5 mg L^{-1} GA_3 medium developed longer shoots (Fig 7 C). Average shoot length in this medium (15.9 mm) was significantly higher than the entries with 0 and 1 mg L^{-1} GA_3 (3.7 and 8.3 mm, respectively; Fig. 6). The GA_3 inhibits meristemoid initiation but is required for shoot development once meristemoids are formed (Moshkov et al., 2008). The timing and duration of treatments will therefore strongly influence the inhibition or promotion of adventitious shoot formation.

Yang et al. (2000) used 0.5 mg L^{-1} GA_3 for shoot elongation in the transformation of grapefruit (*Citrus paradise* Macf.). Oliveira et al. (2009) ahead of shoot-tip grafting of pineapple, sweet orange (*C. sinensis*) and swingle citrumelo (*C.paradise*Macf. × *Poncirustrifoliate* L.), cultured transformed shoots on medium containing 1 mg L^{-1} GA_3 for 3 days. Almeida et al. (2003) transformed natal and Valencia sweet orange using regeneration medium containing 1 mg L^{-1} GA_3 for shoot elongation.

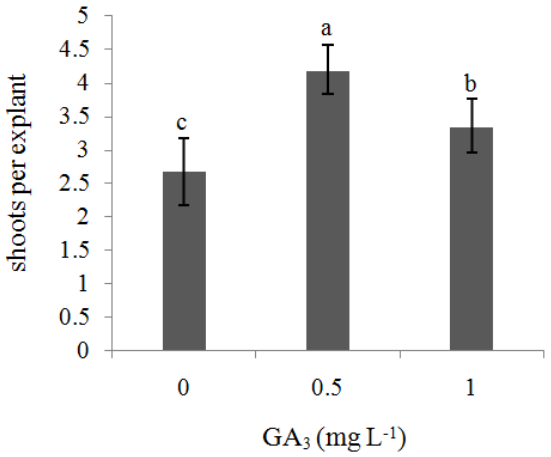

Fig 5. The effects of GA_3 (mg L^{-1}) on the number of developed shoots per explant. Explants extracted from seedlings grown under D+L condition and in the presence of 2.5 mg L^{-1} BAP plus 0.05 mg L^{-1} NAA.

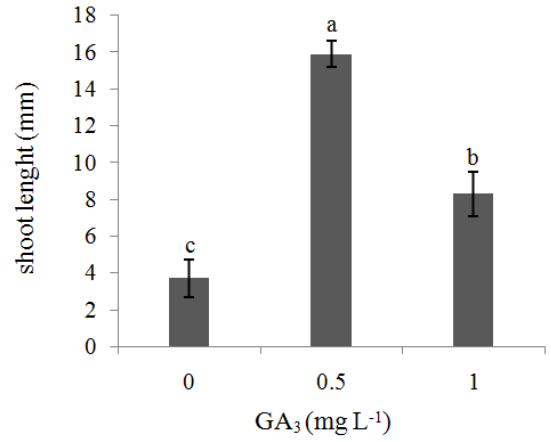

Fig 6. The effects of GA_3 (mg L^{-1}) on length of developed shoots. Explants extracted from seedlings grown at D+L condition and in the presence of 2.5 mg L^{-1} BAP plus 0.05 mg L^{-1} NAA.

Material and Methods

Source of tissue and preparation of explants

Sour orange fruits were collected from Ramsar *Citrus* Research Center, Ramsar, Iran. Seeds were extracted from mature sour orange and air dried at room temperature for 24 hours. Following removal of integuments, the seeds were sterilized in 2.5% sodium hypochlorite solution for 15 minutes by shaking and followed by three times rinsing in sterilized distilled water. Subsequently, seeds were soaked in 75% ethanol for 1 min and rinsed by sterilized distilled water three times under aseptic conditions. Epicotyl segment-derived explants (0.8 - 1.0 cm) were extracted from seedlings (12 to 15 cm in height) and a 1 mm longitudinal incision was made at each end.

Fig 7. Adventitious bud formation (A) and direct shoots organogenesis (B) from epicotyl-originated explants in *C. aurantium*, which explants extracted from seedlings grown at D+L condition and in the presence of 2.5 mg L^{-1} BAP plus 0.05 mg L^{-1} NAA; enhancement of shoots length by the effect of GA_3 (mg L^{-1}) in shoot growth media (C).

Light conditions

Disinfected seeds were cultured in MS medium (Murashige and Skoog, 1962), which supplemented by 30 g L^{-1} sucrose; the medium pH was adjusted to 5.7 before the addition of 8 g L^{-1} agar. The cultured seeds either maintained for 6 weeks in the absolute darkness or cultured in the dark for 4 weeks followed by 10 days in 16-h photoperiod. The cultures were kept under 56 µmol m^{-2} s^{-1} light intensity at 27 ± 2°C for all stages (Almeida et al., 2003).

Pre-culture conditions

In next step, explants extracted from D+L experiment were either pre-cultured in a liquid medium for 1 or 2 days or directly cultured in regeneration medium. Pre-culture liquid medium contains MS salts, B_5 vitamins (Sigma), 500 mg L^{-1} malt extract (Merck), 2.5 mg L^{-1} 6-benzylaminopurine (BAP, Sigma), 0.05 mg L^{-1} Naphthalene acetic acid (NAA, Sigma) and 4.7% w/v sucrose (pH 5.7). The cultures were shacked for 1 and 2 days in absolute darkness at 27 ± 2°C (Almeida et al., 2003). Growth regulators were filter sterilized (0.2 µM) and added to the medium after autoclaving.

Regeneration medium

The explants were transferred to regeneration medium consisting of MS salt, B_5 vitamin, 500mg L^{-1} malt extract, different amount of BAP (0, 1.5, 2.5, 3.5 mg L^{-1}), NAA (0, 0.05, 0.1, 0.2 mg L^{-1}) and 4.7% w/v sucrose (pH 5.7). The explants were maintained at 27 ± 2°C under 16-h photoperiod until they were produced buds. Irradiation of (56 µmol m^{-2} s^{-1}) was provided by cool white fluorescent lamps.

Shoot growth medium

Regenerated explants with 2 to 3 mm long were transferred to a shoot growth medium. It contains MS salts, B_5 vitamins, different amount of Gibberellic Acid (GA_3; Sigma; 0, 0.5, 1 mg L^{-1}), 8 gr L^{-1} agar and the pH was adjusted to 5.7 before autoclaving. The number of adventitious shoots longer than 1 mm from each responsive explant (RE; explants that form bud) was recorded.

Statistical analysis

The experiments related to BAP, NAA and LC were performed based on factorial design. Pre-culture and GA3 related experiments were also performed based on completely randomized design. All experiments were carried out with 3 replications (6-8 explants per petridish). The data were analyzed by ANOVA and the means compared by Dunkan's test at p≤0.01 using SAS 9.1 program. The experiments were performed in 2 biological replications.

Conclusion

In this experiment, we tried to determine the best combination of NAA and BAP, under D+L condition for direct bud induction. Pre-culture of explants left in liquid medium for 1-2 days prior to transforming to regeneration medium has even improved the percentage of RE. Finally, the GA_3 treatment produced effectively large shoots for micro-grafting. We also achieved a high RE (more than 80%), which is a prerequisite for any *C. aurantium* transformation protocol.

Acknowledgments

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