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Regeneration and characterization of plants derived from leaf *in vitro* culture of two sweet orange (*Citrus sinensis* (L.) Osbeck) cultivars

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ABSTRACT

In the current work attempts were made to investigate culture of leaf explants derived from *in vitro* seedlings of two sweet orange (*Citrus sinensis* (L.) Osbeck) cultivars, Bingtangcheng and Valencia. Effects of several factors, including culture medium, lighting condition, explant age and genotype on regeneration response were examined based on three parameters, percentage of explants producing shoots, mean number of shoots per explant and shoot forming capacity. Culture of the explants on shoot-inducing media (SIM) composed of MT salts supplemented with different growth regulators gave rise to disparate shoot regeneration, in which SIM_1 (MT + 0.5 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.1 mg L^{-1} NAA + 3% sucrose + 0.8% agar, pH 5.8) was shown to be the most effective medium for direct induction of shoots from leaf explants. Highly significant difference in the response of shoot bud regeneration was noted between the two cultivars, with Bingtangcheng being more responsive than Valencia. Culture of explants from fully developed leaves led to better shoot regeneration capacity in comparison to undeveloped ones. However, the two lighting conditions used herein did not cause significant difference in shoot regeneration. Phenotypic observation and randomly amplified polymorphic DNA (RAPD) analysis confirmed that all the regenerated plants from both genotypes were genetically identical to their donor plants, suggesting absence of detectable genetic variation in the regenerated plants. The data presented here demonstrated that direct initiation of plants from leaf explants has been successfully accomplished. To our knowledge, this is the first report on direct regeneration of shoots from leaf explants in *Citrus*, which will provide an alternative source for citrus genetic manipulation in the future.

1. Introduction

Citrus is one of the most important and widely grown fruit crops worldwide. During the last decade large stride has been made in citrus industry in terms of increase in production, acreage and yield. Undoubtedly, sustainable development of the citrus industry is mainly dependent on continuous supply of new and improved cultivars. Citrus cultivar improvement via conventional breeding strategies is normally hampered by several factors related to its reproductive biology, such as large tree size, polyembryony, high level of heterozygosity and long juvenile period (Grosser and Gmitter, 1990). Biotechnology is playing an increasingly important role in citrus genetic improvement, in which genetic transformation has been shown to be a powerful strategy for cultivar improvement while maintaining its integrity by adding a single new trait.

Since the first successful report on production of transgenic citrus plants, genetic transformation has been achieved for an array of citrus genotypes or related genus, including sweet orange (*Citrus sinensis*), sour orange (*Citrus aurantium*), trifoliate orange (*Poncirus trifoliata*), citrange (*C. sinensis* × *P. trifoliata*), lemon (*Citrus limon*), lime (*Citrus aurantifolia*), grapefruit (*Citrus paradisi*) and Clementine (*Citrus clementina*) (reviewed by Peña et al., 2007; Cervera et al., 2008). A spectrum of different explants has been tried for this purpose, among which epicotyls and internodal segments of *in vitro* and *in vivo* seedlings and propagated plants are widely used. It is necessary to point out that obtaining adequate segments from a single seedling is not always possible, as in a young seedling only specific part can be efficiently used for *Agrobacterium*-mediated transformation (Moore et al., 1992). Although mature tissues that allow collection of enough explants have been used for transformation in some cases, the high level of

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contamination, low morphogenetic potential and strong recalcitrance to *Agrobacterium tumefaciens* infection largely impeded their wide application (Cervera et al., 1998; Almeida et al., 2003; Cervera et al., 2008). Therefore, searching for other possible explant sources will be of supplementary value for citrus genetic transformation.

It has been shown that in some plants semi-mature and fully developed leaves gave good response of shoot organogenesis *in vitro* (Gu and Zhang, 2005; Raghu et al., 2006). The advantages of using leaves from *in vitro* seedlings lie in the abundant supply of explants and a lower risk of contamination. Leaf discs have been successfully employed as starting explants for *Agrobacterium*-mediated transformation in several plant species (Landi and Mezzetti, 2006; Tsugawa et al., 2004). Unfortunately, so far information is still limited with respect to the induction of organogenesis from leaf or utilization of leaf discs in citrus genetic transformation. Huang et al. (2002) reported that plants were regenerated from leaf-derived callus in pummelo. Later, Almeida et al. (2003) reported that adventitious buds formed from Hamlin sweet orange leaf discs incubated under darkness. However, the organogenesis occurred only in very few leaf explants and shoots regenerated through an intermediate callus phase. To our knowledge, no reports are available on direct shoot regeneration via organogenesis from leaf explants in *Citrus*. Therefore, a question arises, whether citrus plants can be regenerated directly from *in vitro* leaf explants without an intermediate process of callus formation. To answer this question, in the present paper we tested culture of *in vitro* leaves from two sweet orange cultivars, with the intention of developing an efficient and reproducible protocol for multiple shoots induction and direct plant regeneration from *in vitro* leaf explants that can be used for genetic transformation in the future.

2. Materials and methods

2.1. Plant material

Seeds extracted from mature fruits of two sweet orange (*C. sinensis* (L.) Osbeck) cultivars, Valencia (Val) and Bingtangcheng (BTC), were first washed with 1 M NaOH for 15 min, then disinfected in commercial sodium hypochlorite solution (3%) for 20 min, followed by several rinses with sterilized distilled water. After removal of seed coats the seeds were sown in test tubes (150 mm × 25 mm) containing 15 mL of MT (Murashige and Tucker, 1969) basal medium and maintained in the dark for 10–15 d at 26 ± 1 °C before transfer to a 16-h photoperiod (40 μmol m^{-2} s^{-1}). All of the media in the current work, unless otherwise stated, were supplemented with 3% (w/v) sucrose and 0.8% agar (Sigma), and the pH was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min.

2.2. Induction of shoots from leaf discs

Fully developed leaves (FDL) or undeveloped leaves (UDL) excised from 6 to 8 week old Val and BTC seedlings were cut into leaf discs of about 1.0 cm^2 in size, and cultured in 75-mm Petri dishes containing 20 mL of MT basal medium (Control) or four shoot-inducing media (SIM): SIM_1, MT + 0.5 mg L^{-1} BA + 0.5 mg L^{-1} Kinetin + 0.1 mg L^{-1} NAA (Guo et al., 2002); SIM_2, MT + 5.0 mg L^{-1} BA + 5.0 mg L^{-1} Kinetin + 1.0 mg L^{-1} NAA (Liu et al., 2000); SIM_3, MT + 2.0 mg L^{-1} BA + 1.0 mg L^{-1} NAA (Mercier et al., 2003); SIM_4, MS salts (Murashige and Skoog, 1962) + 1.0 mg L^{-1} BA + 0.3 mg L^{-1} NAA (Ghorbel et al., 2000). The explants were positioned in Petri dishes with adaxial surface in contact with the medium. The cultures were sealed with clean parafilm and incubated at 26 ± 1 °C under continuous 16-h photoperiod (40 μmol m^{-2} s^{-1}) or dark/light conditions (6 weeks darkness followed by 16-h photoperiod).

At least 15 leaf explants were placed in each Petri dish, with 5 replicates (Petri dishes) for each treatment, and the experiment was repeated three times. In all of the experiments, the leaf discs were subcultured at 4-week intervals and the cultures were observed visually each week up to 10–12 weeks. Regeneration response was recorded after 3 months of culture initiation on the basis of three parameters, percentage of explants producing shoots (PEPS), mean number of shoots per explant (MNS/E) and shoot forming capacity index (SFC). SFC was calculated according to the equation: SFC = PEPS × MNS/E/100 (Martinez Pulido et al., 1992). For evaluation of the regeneration response, we first observed the effect of each individual factor on all parameters studied and then took into account interaction of all factors on regeneration response of all explants.

2.3. Shoot elongation and rooting

When the regenerated shoots exceeded 1–1.5 cm in height, they were separated from the leaf explants and transferred onto shoot elongation medium composed of MT supplemented with 0.2 mg L^{-1} BA, 0.2 mg L^{-1} IAA and 0.2 mg L^{-1} GA_3. The elongated shoots (2–3 cm) were then placed vertically in root-inducing medium composed of half-strength MT, 0.5 mg L^{-1} NAA, 0.1 mg L^{-1} IBA and 0.5 g L^{-1} activated charcoal.

2.4. Random amplification polymorphic DNA (RAPD) analysis of the regenerated plants

Total DNA was extracted from the leaves as described by Liu et al. (2002). The DNA quality and concentration were analyzed by electrophoresis and spectrophotometry (UV1601 spectrophotometer, Shimadzu, Japan), respectively, followed by dilution to 25 ng μL^{-1} with TE. RAPD analysis with four 10-mer arbitrary primers (A5, A11, A13 and AA18) was performed according to Xu et al. (2007) with minor modification. Twenty microliters of PCR reaction solutions contained 50 ng genomic DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1.0 U *Taq* DNA polymerase, and 0.1 μM of primer. PCR amplifications were carried out in a PTC-200 thermocycler (MJ Research, USA) with the following conditions: 1 cycle of 94 °C, 3 min, 44 cycles of 94 °C, 60 s, 37 °C, 90 s and 70 °C, 2 min and 1 cycle of 72 °C for 10 min. The PCR products were separated in 0.8% agarose gel containing 0.5 μg mL^{-1} ethidium bromide, which was visualized and photographed with UV light.

2.5. Statistical analysis

Experiments were set up in completely randomized design under factorial arrangement. Data were analyzed using STATISTICA (Version 5.5; Stats Soft). Analysis of variance (ANOVA) was used to test the statistical significance, and the significance of differences among means was carried out using Duncan's (1955) multiple range test at $p = 0.05$.

3. Results

3.1. Shoot regeneration from leaf explants

The present investigation showed that shoot buds could be regenerated directly from the citrus leaf explants, regardless of leaf age or genotypes, via organogenesis on four SIMs under two lighting conditions. Enlarged and developed protuberances (or nodular globular structures) were initially observed around the

cutting end of petioles, leaf margins or across the mid-vein within 4 weeks of culture on SIMs (Fig. 1A). Subsequently, the protuberances differentiated into dark green shoot buds (Fig. 1B and C), which underwent normal growth and development (Fig. 1D and E). By contrast, the explants failed to show any response on growth regulator-free MT media under all culture conditions.

3.2. Effect of medium composition on shoot induction

Although shoot induction was accomplished on four SIMs, statistically significant differences in shoot regeneration capacity of leaf explants were noted among the tested SIMs (Table 1). Of the four media tested, explants on SIM_1 first showed shoot initiation (data not shown). Regeneration response of the leaf explants was evaluated based on three parameters, percentage of explants producing shoots (PEPS), mean number of shoots per explant (MNS/E) and shoot forming capacity index (SFC). When only results from culture on the media were compared, regeneration response of the leaf discs on SIM_1 was the best irrespective of other factors (data not shown). As is shown in Table 1, culture of the leaf explants from both genotypes on SIM_1 gave rise to the highest MNS/E (4.29 and 3.16 for BTC and Val, respectively), PEPS (69.33% and 60.00% for BTC and Val, respectively) and SFC (3.04% and 1.89% for BTC and Val, respectively). Meanwhile, MNS/E, PEPS and SFC of the cultured leaf discs on SIM_1 were always higher than on other media in most cases, with the exceptions of some undeveloped leaf explants on SIM_3 under dark/light condition (Table 1). Culture of the leaf explants on SIM_3 and SIM_4 was slightly inferior to that on SIM_1, but both of them produced nearly similar results. SIM_2 proved to be the worst medium for organogenic response of the

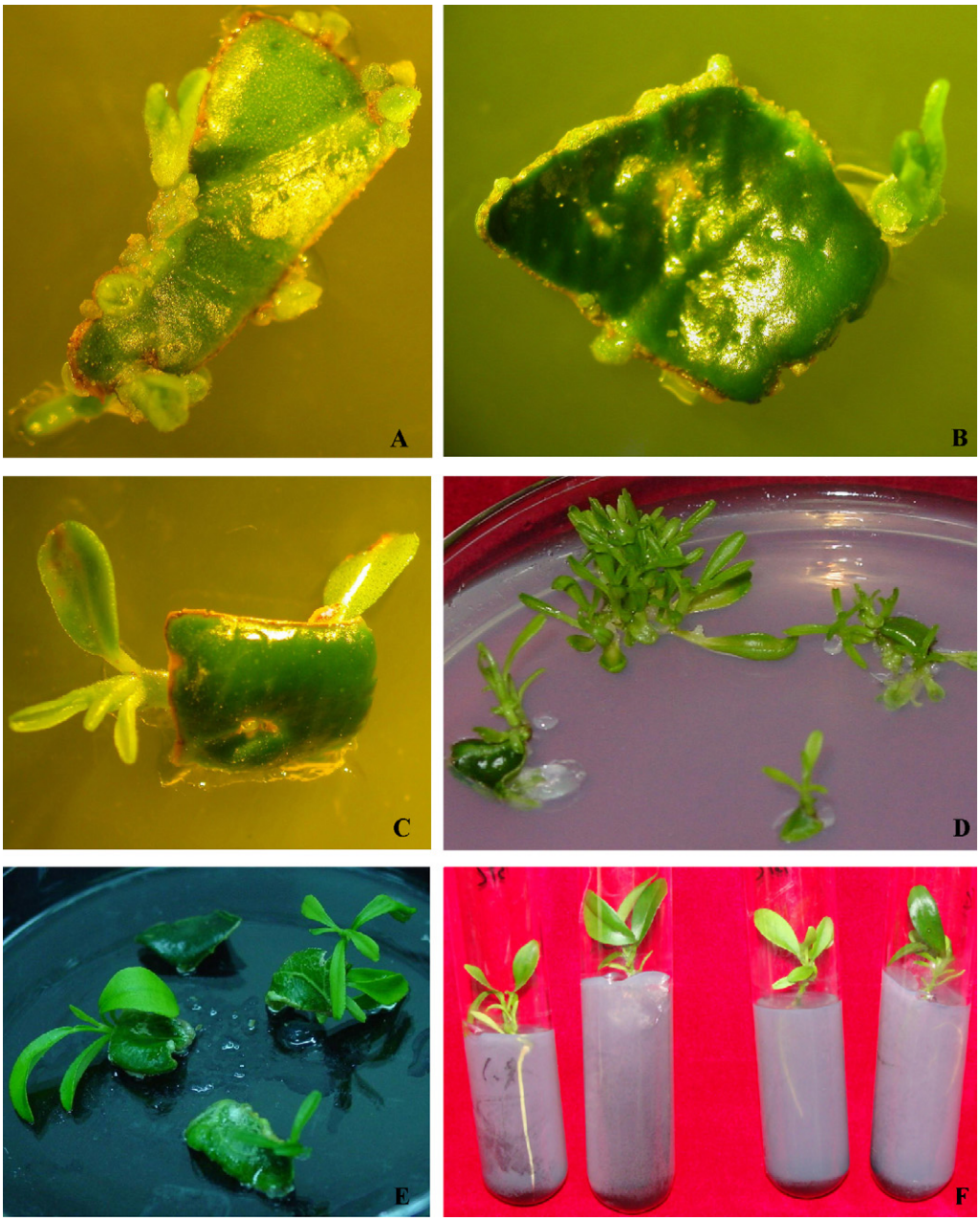

Fig. 1. Direct induction of adventitious shoot buds and plant regeneration via culture of leaf explants from *in vitro* citrus seedlings. (A) Emergence of shoot buds from the cutting edge of a leaf explant. (B and C) A leaf explant with shoot bud from the surface contacting the medium. (D) A photo showing high frequency of regeneration of multiple shoots from the leaf explant. (E) Leaf explants with well-developed shoot. (F) Rooted plants.

Table 1
Regeneration response of Bingtangcheng (BTC) and Valencia (Val) leaf explants of different age on four shoot-inducing media (SIM) under either light or dark/light conditions, based on three parameters, mean number of shoots per explant (MNS/E), percentage of explants producing shoots (PEPS) and shoot forming capacity index (SFC).

Cultivar	Medium	MNS/E				PEPS (%)				SFC (%)			
		Dark/light		Light		Dark/light		Light		Dark/light		Light	
		FDL	UDL	FDL	UDL	FDL	UDL	FDL	UDL	FDL	UDL	FDL	UDL
BTC	SIM_1	4.29 a	1.97 ef	3.59 ab	3.04 bc	69.33 a	41.33 bcd	57.33 ab	46.67 bc	3.04 a	0.79 defg	2.09 b	1.43 bcd
	SIM_2	1.40 fg	1.13 g	1.28 fg	1.23 g	26.67 def	22.67def	18.67 f	17.33 f	0.36 fg	0.27 g	0.24 g	0.23 g
	SIM_3	2.71 cd	2.46 cde	2.42 cde	2.68 cd	48.00 bc	40.00 bcde	41.33 bcd	40.00 bcde	1.32 cde	1.01 def	1.00 def	1.05 cde
	SIM_4	2.96 bc	2.17 de	2.41 cde	1.81 efg	57.33 ab	30.67 cdef	54.67 ab	45.33 bc	1.71 bc	0.67 efg	1.29 cde	0.79 defg
Val	SIM_1	3.16 a	1.65 defg	2.84 ab	1.86 def	60.00 a	30.67 de	49.33 abc	41.33 cd	1.89 a	0.51 de	1.41 b	0.80 cd
	SIM_2	1.03 g	1.15 fg	1.13 fg	1.22 efg	18.67 ef	18.67 ef	14.67 f	16.00 f	0.20 e	0.21 e	0.17 e	0.20 e
	SIM_3	2.34 bcd	2.34 abcd	2.77 abc	2.11 bcd	49.33 abc	46.67 bc	48.00 abc	37.33 cd	1.15 bc	1.09 bc	1.31 b	0.75 cd
	SIM_4	2.17 bcd	1.96 cde	2.82 ab	1.89 def	54.67 ab	38.67 cd	46.67 bc	38.67 cd	1.13 bc	0.77 cd	1.35 b	0.73 cd

Data were means from three repetitions, each containing at least 15 leaf explants in five Petri dishes (replicates). Means followed by the same letters, in the same parameter, were not significantly different among the investigated factors (medium, leaf age and lighting conditions) according to Duncan's multiple range test ($p = 0.05$). FDL and UDL are fully developed leaves and undeveloped leaves, respectively.

leaf explants under any circumstances, which implied that this medium may not be suitable for inducing organogenesis from leaf. High frequency of shoot bud regeneration and better vigor and development were achieved in subsequent subcultures on SIM_1 (data not shown).

3.3. Effect of cultivar on shoot induction

The two cultivars used herein showed different regeneration response although regeneration of shoots occurred in both of them (Table 1). Independently of the medium and lighting conditions used, when only the cultivar type was considered, BTC presented on average significantly higher MNS/E (2.34) and SFC (0.96%) than Val (2.03 and 0.77%, respectively), whereas no significant difference was observed in PEPS (41.08% and 38.08% for BTC and Val, respectively). A close comparison between these two genotypes, even if all of the factors were taken into consideration, also revealed that culture of leaf explants from BTC showed higher MNS/E, SFC and PEPS than Val in most cases (Table 1), which shows that BTC was more productive than Val.

3.4. Effect of leaf age on shoot regeneration

Adventitious shoots were regenerated from explants excised from both fully developed and undeveloped leaves. However, marked difference was observed between leaf explants of different age. If data were only examined based on leaf age, explants from fully developed leaves had on average (44.67%, 2.46% and 1.10%, respectively and correspondingly) significantly higher PEPS, MNS/E and SFC than those from undeveloped leaves (34.50%, 1.92% and 0.66%, respectively), suggesting that the fully developed leaves were superior in shoot regeneration capacity in comparison to the undeveloped ones. Likewise, when all of the factors were evaluated simultaneously, the three parameters of fully developed leaf explants were larger than the undeveloped leaves in most cases (Table 1), implying better regeneration capacity in the former.

3.5. Effect of lighting conditions on shoot induction

Under both types of lighting conditions, adventitious shoots initiated from the cultured leaf explants of both cultivars. It was noted that no significant difference in the regeneration response was observed among the cultures, except MNS/E of undeveloped leaf of BTC and SFC of full developed leaf from both BTC and Val on SIM_1 (Table 1), which showed that the lighting conditions may have little impacts on the regeneration of leaf explants in our system.

3.6. Rooting of elongated shoots and RAPD analysis of regenerated plants

When the elongated shoots reached 2–3 cm in height they were transferred to root-inducing medium. Roots were observed as early as 3 weeks after the transfer, while most of the shoots developed roots within 7 weeks. Shoots of BTC had a better rooting response (67.62%) compared with those of Val (61.65%). Finally, rooted plantlets (Fig. 1F) were successfully recovered from both BTC and Val, despite a slightly different rooting response between the two cultivars. *In vitro* plantlets grew actively during the acclimatization process and viable plants in pots were ultimately established in greenhouse (Fig. 2), which were morphologically similar to the mother plant.

In order to confirm whether somaclonal variation was detectable in the regenerated plants RAPD was employed to analyze the genetic fidelity of four plants randomly selected from the regenerated population of both cultivars. Four polymorphic primers (A5, A11, A13 and AA18) that have been screened earlier were used for this purpose. As can be seen in Fig. 3, the regenerated plants shared the same banding patterns as those of the donor plants providing leaf explants, implying that they were possibly genetically identical to each other.

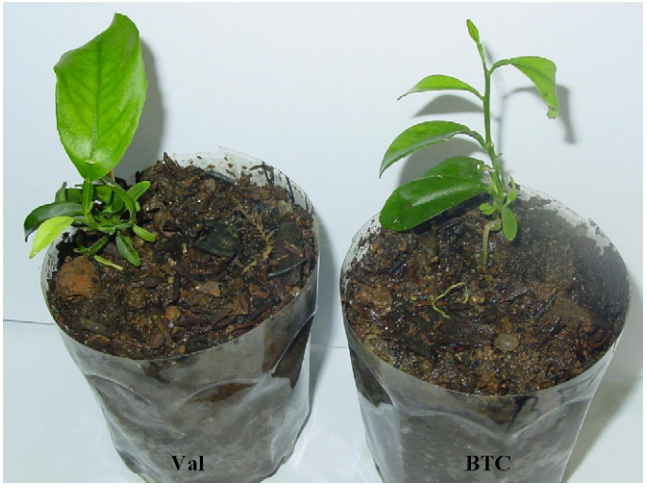

Fig. 2. Plants grown in soil pots that were regenerated from *in vitro* culture of leaf explants of Valencia (Val) and Bingtangcheng (BTC).

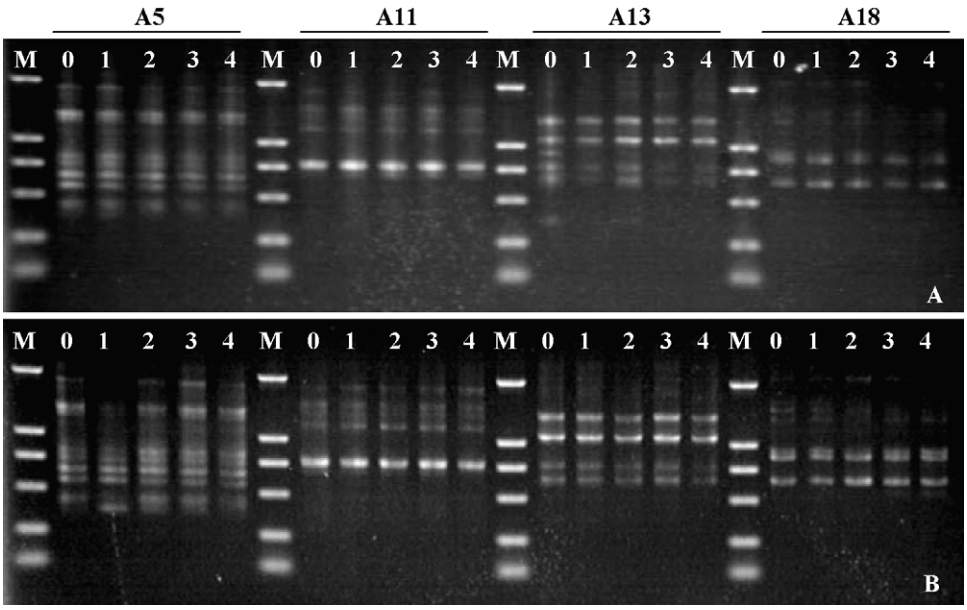

Fig. 3. RAPD analysis of the regenerated plants in comparison to their respective donor plants. (A and B) Banding profiles of the regenerated plants (1–4) and the donor plant (0) of Bingtangcheng (A) and Valencia (B), revealed by four arbitrary primers, A5, A11, A13 and AA18. M, a marker used to show the band size.

4. Discussion

In the current work we induced adventitious shoots directly via organogenesis from *in vitro* cultured leaf explants of the two sweet orange cultivars. To our knowledge, this constitutes the first report of plant regeneration via direct organogenesis from leaf explants in *Citrus*. The entire process could be completed without an intervening callus formation. This point is of major importance because plants produced by direct organogenesis may exhibit greater genetic stability than those produced from callus (Lee and Phillips, 1988; Karam and Al-Majathoub, 2000).

It is well known that *in vitro* regeneration of an explant is always affected by internal or external factors. Therefore, in this work we examined regeneration response of the leaf explants in various culture conditions. Culture medium has been shown to be a critical factor for induction of organogenesis in plant tissue culture. In the current work we used four different media to induce shoots from citrus leaf explants. These media, with various combinations of cytokinin and auxin, triggered direct shoot organogenesis from the leaf explants, where SIM_1 proved to be the best one in terms of MNS/E, PEPS and SFC. SIM_1 was different from SIM_3 and SIM_4 as it contained Kinetin, a kind of cytokinin, in addition to BA and NAA. The importance of cytokinin for organogenesis has been studied in many plant species (Pereira et al., 2000; Guo et al., 2005; Zhang et al., 2008), and was considered as an exclusive element for shoot formation. However, it was noted that SIM_2 containing higher concentration of Kinetin gave rise to the lowest regeneration response, which may be ascribed to toxic effects of high concentration of the hormone, as has been reported for other type of cytokinin (Wang et al., 2007). Our results agreed with earlier reports in which low concentration of cytokinin was more effective for induction of shoots (Sahoo et al., 1997).

Apart from the induction medium, age of the leaf providing the explants was found to be another important determinant for regeneration response, which may be ascribed to different physiological status of the explants at various developmental stage. In fact, a variety of previous work on other plant species has also demonstrated that the age of explant donors affected regeneration, although the exact influence remains a matter of debate and varies among plant species (Grant and Hammatt, 2000). Our results suggested that the fully developed leaves of *in vitro* seedlings showed better regeneration capacity than the undeveloped ones, which is consistent with the findings of Lane et al. (1998), Rout (2002) and Mahalakshmi et al. (2006), whereas Perez-Tornero et al. (2000) and Gu and Zhang (2005) presented the opposite conclusion, implying that influence of explant age may be dependent upon other factors that are not deciphered as yet. In addition, influence of explant age also depended on the explant source (initiating materials) used for *in vitro* culture. For example, the regeneration response of internodal explants differed from that of leaf reported here, since juvenile/young internodes were more responsive than the adult/mature ones (Cervera et al., 1998; Rodriguez et al., 2008). However, it is worth mentioning that we only focused on leaves from *in vitro* seedlings in this work. In the future, more attempts could be made to investigate if our results could be extended to leaves from adult citrus plants grown in greenhouse, as they can provide more initiating materials and regenerated plants from these sources, and allowed a faster agronomic evaluation (Almeida et al., 2003).

Significant differences in shoot induction were also detected between the two sweet orange cultivars. Under the same culture cues, BTC was more responsive than Val, indicating the presence of genotype/cultivar-specific reactions. Cultivar-dependent variation in shoot regeneration capacity has been previously reported in several plants including pear (Caboni et al., 1999), peach (Gentile et al., 2002), black berry (Espinosa, 2006), strawberry (Landi and Mezzetti, 2006; Mohamed et al., 2007) and citrus (Almeida et al., 2003; Rodriguez et al., 2008). Previous work and ours demonstrated that regeneration potential of the explants was tightly correlated with genetic background of the stock plants, which may be due to different levels of endogenous metabolites and/or hormones that are involved in regeneration process.

Some previous work has provided evidence showing that photoperiod posed significant impact on regeneration (Moreira-Dias et al., 2000; Gu and Zhang, 2005; Espinosa, 2006). Photoperiod might affect the regeneration frequency or regeneration pattern (direct or indirect). For example, Almeida et al. (2003) presented data showing that indirect regeneration via callus was detected

under darkness, while under 16-h photoperiod only direct induction of shoot buds took place from internodal segments. In most cases, culture of the explants in darkness for a certain period seemed to be beneficial for shoot regeneration, as has been reported by Almeida et al. (2003), Gu and Zhang (2005) and Espinosa (2006). Exposure to a dark period may possibly modify the proportion of endogenous hormones like cytokinins and auxins, which interact more reasonably with exogenously applied growth regulators in the culture medium, leading to promotion of shoot regeneration (Miguel et al., 1996; Gentile et al., 2002). Nevertheless, it was noted that in our work the two light regimes seemed to have little bearing on the regeneration response based on the three parameters and morphological observation. The reason for this discrepancy remains unclear and more work is needed to clarify this point.

Generally, it is important to ensure that the regenerants were genetically true-to-type of their donor plants with respect to genetic fidelity. In order to know if there is any aberration in the regenerated plants, RAPD, a marker that has been revealed to be a potential marker for distinguishing genetic variation (Piccioni et al., 1997; Raimondi et al., 2001), was employed for this purpose, which demonstrated that all of the regenerants showed genetic stability in our regeneration system. Therefore, we can conclude that direct regeneration from leaf did not induce any somaclonal variation that has been depicted in other explants-mediated culture (Cassells and Curry, 2001; Šušek et al., 2002).

Taken together, our data showed that plant regeneration through *in vitro* leaf explant has been successfully achieved, although the efficiency was affected by different factors. The *in vitro* regeneration of adventitious shoots from leaf offers a potential alternative for citrus genetic transformation and clonal propagation, which is currently underway in our laboratory.

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References

Almeida, W.A.B., Mourao-Filho, F.A.A., Pino, L.E., Boscariol, R.L., Rodriguez, A.P.M., Mendes, B.M.J., 2003. Genetic transformation and plant recovery from mature tissues of *Citrus sinensis* L. Osbeck. Plant Sci. 164, 203–211.

Caboni, E., Tonelli, M.G., Lauri, P., Angeli, S.D., Damiano, C., 1999. In vitro shoot regeneration from leaves of wild pear. Plant Cell Tiss. Org. Cult. 59, 1–7.

Cassells, A.C., Curry, R.F., 2001. Oxidative stress and physiological, epigenetic and genetic variability in plant tissue culture: implications for micropropagators and genetic engineers. Plant Cell Tiss. Org. Cult. 64, 145–157.

Cervera, M., Juárez, J., Navarro, A., Pina, J.A., Durán-Vila, N., Navarro, L., Peña, L., 1998. Genetic transformation and regeneration of mature tissues of woody fruit plants bypassing the juvenile stage. Transgenic Res. 7, 51–59.

Cervera, M., Navarro, A., Navarro, L., Peña, L., 2008. Production of transgenic adult plants from clementine mandarin by enhancing cell competence for transformation and regeneration. Tree Physiol. 28, 55–66.

Duncan, D.B., 1955. Multiple range and a multiple *F* tests. Biometrics 11, 1–42.

Espinosa, A.C., 2006. Adventitious shoot regeneration and rooting of *Prunus serotina* in vitro cultures. HortSci. 41, 193–201.

Gentile, A., Monticelli, S., Damiano, C., 2002. Adventitious shoot regeneration in peach [*Prunus persica* (L) Batch]. Plant Cell Rep. 20, 1011–1016.

Ghorbel, R., Dominguez, A., Navarro, L., 2000. High efficiency genetic transformation of sour orange (*Citrus aurantium*) and production of transgenic trees containing the coat protein gene of citrus tristeza virus. Tree Physiol. 20, 1183–1189.

Grant, N.J., Hammatt, N., 2000. Adventitious shoot development from wild cherry (*Prunus avium* L.) leaves. New Forest. 20, 287–295.

Grosser, J.W., Gmitter Jr., F.G., 1990. Protoplast fusion and *Citrus* improvement. Plant Breed. Rev. 8, 339–374.

Gu, X.F., Zhang, J.R., 2005. An efficient adventitious shoot regeneration system for Zhanhua winter jujube (*Zizyphus jujube* Mill.) using leaf explants. Plant Cell Rep. 23, 775–779.

Guo, W.W., Cheng, Y.J., Deng, X.X., 2002. Regeneration and molecular characterization of intergeneric somatic hybrids between *Citrus reticulata* and *Poncirus trifoliata*. Plant Cell Rep. 20, 829–834.

Guo, D.P., Zhu, Z.J., Hu, X.X., Zeng, S.J., 2005. Effect of cytokinins on shoot regeneration from cotyledon and leaf segment of mustard (*Brassica juncea* var. tsatsai). Plant Cell Tiss. Org. Cult. 83, 123–127.

Huang, T., Peng, S., Dong, G., Zhang, L., Li, G., 2002. Plant regeneration from leaf-derived callus in *Citrus grandis* (pummelo): effects of auxin in callus induction medium. Plant Cell Tiss. Org. Cult. 69, 141–146.

Karam, N.S., Al-Majathoub, M., 2000. Direct shoot regeneration and microtuberization in wild *Cyclamen persicum* Mill. using seedling tissue. Sci. Hortic. 86, 235–246.

Landi, L., Mezzetti, B., 2006. TDZ, auxin and genotype effects on leaf organogenesis in *Fragaria*. Plant Cell Rep. 25, 281–288.

Lane, W.D., Iketani, H., Hayashi, T., 1998. Shoot regeneration from cultured leaves of Japanese pear (*Pyrus pyrifolia*). Plant Cell Tiss. Org. Cult. 54, 9–14.

Lee, M., Phillips, R.L., 1988. The chromosomal basis of somaclonal variation. Ann. Rev. Plant Physiol. Plant Mol. Biol. 39, 413–437.

Liu, J.H., Hu, C.G., Deng, X.X., 2000. Production of intergeneric somatic hybrid plants via protoplast electro fusion in citrus. Acta Bot. Sin. 41, 1177–1182.

Liu, J.H., Pang, X.M., Cheng, Y.J., Meng, H.J., Deng, X.X., 2002. Molecular characterization of the nuclear and cytoplasmic genomes of intergeneric diploid plants from cell fusion between *Microcitrus papuana* and rough lemon. Plant Cell Rep. 21, 327–332.

Mahalakshmi, L.S., Leela, T., Kumar, S.M., Kumar, B.K., Naresh, B., Devi, P., 2006. Enhanced genetic transformation efficiency of mungbean by use of primary leaf explants. Curr. Sci. 91, 93–99.

Martinez Pulido, C., Harry, I.S., Thorpe, T.A., 1992. Optimization of bud induction in cotyledonary explants of *Pinus canariensis*. Plant Cell Tiss. Org. Cult. 29, 247–255.

Mercier, H., Souza, B.M., Kraus, J.E., Hamasaki, R.M., Sotta, B., 2003. Endogenous auxin and cytokinin contents associated with shoot formation in leaves of pineapple cultured in vitro. Braz. J. Plant Physiol. 15, 107–112.

Miguel, C.M., Duart, P., Olieveira, L., 1996. Shoot regeneration from adventitious buds induced on juvenile and adult almond (*Prunus dulcis* Mill.) explants. In Vitro Cell. Dev. Biol. Plant 32, 148–153.

Mohamed, F.H., Beltagi, M.S., Mona, A., Ismail, M.A., Omar, G.F., 2007. High frequency, direct shoots regeneration from greenhouse-derived leaf disks of six strawberry cultivars. Pak. J. Biol. Sci. 10, 96–101.

Moore, G.A., Jacono, C.C., Neidigh, J.L., Lawrence, S.D., Cline, K., 1992. *Agrobacterium*-mediated transformation of *Citrus* stem segments and regeneration of transgenic plants. Plant Cell Rep. 11, 238–242.

Moreira-Dias, J.M., Molina, R.V., Bordon, Y., Guardiola, J.L., Garcia-Luis, A., 2000. Direct and indirect shoot organogenesis pathways in epicotyl cuttings of Troyer citrange. Sci. Hortic. 85, 103–110.

Murashige, T., Skoog, F., 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 13, 473–497.

Murashige, T., Tucker, D.P.H., 1969. Growth factor requirement of citrus tissue culture. In: Proceedings of the 1st Internatinoal Citrus Symposium on Riverside Univ. Calif. pp. 1155–1169.

Peña, L., Cervera, M., Fagoaga, C., Romero, J., Juarez, J., Pina, J.A., Navarro, L., 2007. *Citrus*. In: Pua, E.C., Davey, M.R. (Eds.), Biotechnology in Agriculture and Forestry, Vol. 60, Transgenic Crops V. Springer-Verlag Berlin, Heidelberg, New York, pp. 35–50.

Pereira, A.M., Bertoni, B.W., Appezzato-da-Gloria, B., Araujo, A.R.B., Januario, A.H., Lourenco, M.V., Franca, S.C., 2000. Micropropagation of *Pothomorphe umbellate* via direct organogenesis from leaf explants. Plant Cell Tiss. Org. Cult. 60, 47–53.

Perez-Tornero, O., Egea, J., Vanoostende, A., Burgos, L., 2000. Assessment of factors affecting adventitious shoot regeneration from in vitro cultured leaves of apricot. Plant Sci. 158, 61–70.

Piccioni, E., Barcaccia, G., Falcinelli, M., Standardi, A., 1997. Estimating alfalfa somaclonal variation in axillary branching propagation and indirect somatic embryogenesis by RAPD fingerprinting. Intl. J. Plant Sci. 158, 556–562.

Raghu, A.V., Geetha, S.P., Martin, G., Balachandran, I., Ravindran, P.N., 2006. Direct shoot organogenesis from leaf explants of *Embelia ribes* Burm. f.: a vulnerable medicinal plant. J. Forest Res. 11, 57–60.

Raimondi, J.P., Masuelli, R.W., Camadro, E.L., 2001. Assessment of somaclonal variation in asparagus by RAPD fingerprinting and cytogenetic analyses. Sci. Hortic. 90, 19–29.

Rodriguez, A., Cervera, M., Peris, J.E., Peña, L., 2008. The same treatment for transgenic shoot regeneration elicits the opposite effect in mature explants from two closely related sweet orange (*Citrus sinensis* (L.) Osb.) genotypes. Plant Cell Tiss. Org. Cult. 93, 97–106.

Rout, G.R., 2002. Direct plant regeneration from leaf explants of plumbago species and its genetic fidelity through RAPD markers. Ann. Appl. Biol. 140, 305–313.

Sahoo, Y., Pattnaik, S.K., Chand, P.K., 1997. In vitro clonal propagation of medicinal plant *Ocimum basilicum* L. (Sweet basil) by axillary shoot proliferation. In Vitro Cell. Dev. Biol. Plant 33, 293–296.

Šušek, A., Javornik, B., Bohanec, B., 2002. Factors affecting direct organogenesis from flower explants of *Allium giganteum*. Plant Cell Tiss. Org. Cult. 68, 27–33.

Tsugawa, H., Kagami, T., Suzuki, M., 2004. High-frequency transformation of *Lobelia erinus* L. by *Agrobacterium*-mediated gene transfer. Plant Cell Rep. 22, 759–764.

Wang, B., Peng, D.X., Liu, L.J., Sun, Z.H., Zhang, N., Gao, S.M., 2007. An efficient adventitious shoot regeneration system for ramie (*Boehmeria nivea* Gaud) using thidiazuron. Bot. Stud. 48, 173–180.

Xu, X.Y., Hu, Z.Y., Li, J.F., Liu, J.H., Deng, X.X., 2007. Asymmetric somatic hybridization between UV-irradiated *Citrus unshiu* and *C. sinensis*: regeneration and characterization of hybrid shoots. Plant Cell Rep. 26, 1263–1273.

Zhang, Y., Zhou, J., Wu, T., Cao, J., 2008. Shoot regeneration and relationship between organogenic capacity and endogenous hormonal contents in pumpkin. Plant Cell Tiss. Org. Cult. 93, 323–331.