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Organogenesis of *Citrus* Rootstocks Using Mature Explants

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Abstract

Organogenesis of the commercial Citrus rootstocks *P. trifoliata*, *C. aurantium* and Swingle citrumelo was studied, using mature shoot and leaf explants. In *P. trifoliata*, the number of sprouted buds per shoot explant increased at both 0.5 and 2.0 mg L^{-1} BA concentrations; shoot number increased significantly at 2 mg L^{-1} BA, while shoot length increased significantly at both BA concentrations. In *C. aurantium* explants, shoot number increased when 2.0 mg L^{-1} BA were added in the medium, and shoot length increased using 0.5 mg L^{-1} BA. In all the treatments of *P. trifoliata* and *C. aurantium* explants, callus formation was observed at their base. On leaf explants, it was observed callus formation of different colour; yellow, yellow - brown, brown and green. In *C. aurantium* the highest callus formation was observed using 5.500 mg L^{-1} TDZ. In *P. trifoliata* the highest percentage of callus formation were observed using 2.0 or 5.0 mg L^{-1} NAA, with 0.5 mg L^{-1} BA, and the callus was green. In Citrumelo leaf explants, TDZ caused the maximum callus formation, alone or in combination with BA. So, *in vitro* organogenesis of citrus rootstocks can be accomplished using mature tissues, in a successful and quick way.

Keywords: callus formation, growth regulators, *P. trifoliata*, *C. aurantium*, Swingle citrumelo, BA

1. Introduction

The need for genetically stable rootstocks is essential. *Poncirus trifoliata* L. is a valuable citrus rootstock and it is also used to produce citrus hybrids. It has a remarkable resistance to cold and to some specific diseases and it is also a suitable rootstock for replanting. Sour orange (*Citrus aurantium* L.) is one of the citrus rootstocks which are resistant to salinity, high pH, calcareous soils and root rot *(Phytophthora spp)* (Ben-Hayyim & Moore, 2007). Swingle citrumelo is a hybrid obtained after pollination of grapefruit (*Citrus paradisi*) flowers with *P. trifoliata* pollen. It performs well as an alternative to *P. trifoliata* and its hybrids. Its resistance to *Phytophthora spp*, nematodes (*Tylenchulus semipenetrans*), and to low temperatures is similar to or better than that of other common trifoliate types. In addition, Citrumelo has shown more tolerance to citrus blight (Gmitter *et al.*, 2009). To date, the rate of shoot proliferation from citrus mature tissues remains relatively low compared to juvenile material and little is known about the effects of cultivars, explant type, growth regulators and nutrient substrate on *in vitro* culture of citrus mature tissues (Marutani-Hert *et al.*, 2012; Tallon *et al.*, 2013).

Availability of efficient *in vitro* plantlet regeneration protocol is crucial for efficient micropropagation. During the last decades, it became possible to regenerate whole plantlets from various citrus explants, among which epicotyls and internodal segments have been found to be more satisfying (Perez-Tornero *et al.*, 2010; Tallon *et al.*, 2013).

For many woody species, mature tissue is not suitable material for *in vitro* organogenesis. In Citrus, the faster propagation is commonly obtained by using juvenile tissues (Pérez-Molphe-Balch & Ochoa-Alejo, 1997) and more specifically using tissues derived from *in vitro* germinated seeds (Curtis & Mirkov, 2012). However, due to juvenile characteristics of the regenerating plants, it requires several years for maturation, before they can be evaluated for horticultural and commercial traits (Curtis & Mirkov, 2012).

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Furthermore, due to difficulty of producing seeds in seedless citrus cultivars, availability of a protocol for plantlet regeneration from mature explants would be of great significance. The plants lacking juvenile characteristics would be ready for field screening in a shorter period, thus allowing a more rapid selection process (Kobayashi *et al.*, 2003). Although mature explants have been used for micropropagation in some cases, the high rate of contamination, the low morphogenetic capacity and poor shoot rooting largely impeded their wide application (George, 1993; Curtis & Mirkov, 2012). Lastly, few protocols developed in citrus micropropagation using mature explants but little is known about the effects of genotype, explant type and growth regulators on *in vitro* culture of citrus mature tissues (Pérez-Molphe-Balch & Ochoa-Alejo, 1997; Bordón *et al.*, 2000; Kobayashi *et al.*, 2003; Pérez-Tornero *et al.*, 2010; Marutani-Hert *et al.*, 2012; Tallon *et al.*; 2013).

Many studies indicated that some plant species produce shoots and roots from callus. In this process, 6-benzyl-adenine (BA) is the most efficient synthetic cytokinin (Carimi & De Pasquale, 2003). Furthermore, BA is an important agent for bud sprouting and proliferation of new shoots (Tzatzani *et al.*, 2013; Esmaeilnia & Dehestani, 2015) and is used more than the other cytokinins mainly for citrus shoot proliferation (Carimi & De Pasquale, 2003). Thidiazuron (TDZ, N-phenyl-N-1,2,3-thidiazol-5-ylurea) also acts like cytokinin and it is a potential regulator of morphogenetic responses in micropropagation (Corredoira *et al.*, 2008), regeneration and shoot formation from leaves, cotyledons and hypocotyls (Sanikhani *et al.*, 2006). Its effectiveness has been confirmed even in mature tissues (Mroginski *et al.*, 2004; Corredoira *et al.*, 2008).

The findings of our study would have an important impact on producing by micropropagation shoots lacking a juvenile phase, thus accelerating the evaluation of the regenerated plantlets. Furthermore, using mature tissues would be a preferable way, since mature explants can be collected throughout the year and in bulk quantities.

2. Material and Methods

2.1 Plant material, culture medium, growth conditions and reagents

In the first experiment, one-year old shoot segments (2.5-3.0 cm long), of *P. trifoliata* L. and *C. aurantium* collected on spring, were used as explants. The three-year-old mother plants were grown in a greenhouse. The mature shoot segments were cultured in an MS (Murashige & Skoog, 1962) medium supplemented with 0, 0.5 and 2.0 mg L^{-1} BA. In the second experiment one-year old leaves of *P. trifoliata*, *C. aurantium* and Swingle citrumelo were used. The leaves were etched lightly to their lower side with a razor blade and were placed horizontally on the substrate. They were cultured in an MS medium supplemented with growth regulators. The following treatments were included in the experiment: Control, BA (0.5, 1, 2, 3, 4 mg L^{-1}), NAA (2, 5, 8 mg L^{-1}), NAA/BA (2/0.5, 5/0.5, 8/0.5 mg L^{-1}), IAA/BA (0.2/2, 0.5/2, 0.2/3, 0.5/3 mg L^{-1}, TDZ (0.011, 0.110, 1.100, 3.300, 5.500 mg L^{-1}) and TDZ (0.011, 0.110, 1.100, 3.300 and 5.500 mg L^{-1}) + 0.5 mg L^{-1} BA.

The explants were transplanted to glass tubes of 25 x 100 mm. Each tube contained 10 ml of modified MS culture medium, supplemented with 30 g L^{-1} sucrose, 6 g L^{-1} agar-agar and the previous concentrations of the tested growth regulators. The pH of the culture medium was adjusted to 5.8 value with 0.01 N NaOH before autoclaving for 15 minutes at 121° C. All plant growth regulators were added before autoclaving. Each treatment included 10 replications (tubes), maintained in a growth chamber at 25±1° C, 16 hours photoperiod and light intensity 45 μmol m^{-2} s^{-1} (Philips TLD 54/36W) for 8 weeks.

2.2 Statistical analysis

At the termination of the experiment, after 8 weeks, the number of sprouted buds on mature shoots, the number of new shoots, shoot length and the percentage of leaves formed callus were measured. The colour of callus of mature leaves was also recorded. The whole experiment was repeated twice and the results presented are the means. Data were analyzed by one-way analysis of variance (ANOVA) and the means differing significantly were compared by using the Duncan's multiple range test at $p \leq 0.05$.

3. Results and Discussion

3.1 Organogenesis from mature shoot explants

For promoting organogenesis from *P. trifoliata* and *C. aurantium* mature shoot explants BA was used, as the most appropriate and widespread growth regulator for Citrus shoot proliferation (Carimi & De Pasquale, 2003; Esmaeilnia & Dehestani, 2015).

The number of sprouted buds of *P. trifoliata* increased at both 0.5 and 2 mg L^{-1} BA (Table 1). The shoot number per explant increased after the treatment with 2 mg L^{-1} BA. In contrast, shoot length was not significantly affected by inclusion in the substrate of BA.

Table 1. Organogenesis of *P. trifoliata* L. and *C. aurantium* L. mature shoot explants *in vitro*. The effect of BA on sprouted buds, shoot mumber, shoot height, and percentage of callus formation per explant.

Treatments (mg L^{-1} BA)	Sprouted buds number	Shoot number	Shoot height (mm)	Percentage of callused explants
	P. trifoliata L.			
0	1.9 a	2.1 a	6.7 a	17
0.5	2.9 b	3.6 a	7.4 b	100
2.0	3.0 b	5.8 b	8.2 b	100
	C. aurantium L.			
0	1.3 a	1.4 a	6.3 a	20
0.5	1.2 a	1.2 a	9.8 b	100
2.0	1.5 a	2.2 b	7.5 b	100

* Means followed by the same letter in the same column and the same rootstock are not significantly different according to Duncan's multiple range test ($p \leq 0.05$).

Concerning the genotype *C. aurantium*, addition of BA in the substrate, did not affect the number of sprouted buds, while shoot number increased when 2.0 mg L^{-1} BA were added in the medium, and shoot length increased with 0.5 mg L^{-1} BA. In all treatments of *P. trifoliata* and *C. aurantium* shoot explants, callus formation was observed at their base. The percentage of explants formed callus with BA was 100%.

The BA concentrations used were up to 2 mg L^{-1} and not higher, as the detrimental effect of high concentrations of BA has been previously demonstrated for a number of Citrus genotypes including sour orange (Molina *et al.* 2007, Cervera *et al.* 2008, Tallon *et al.* 2013, Esmaeilnia & Dehestani, 2015). Furthermore, callus formation is another parameter of organogenesis related to BA, that produced in our explants. Also, when high concentrations of BA were used, shoot length was drastically decreased inappropriate for micropropagation and a mass of green hard callus emerged at the cut surfaces (Esmaeilnia & Dehestani, 2015).

Similar to our results, Kotsias & Roussos (2001) observed that in explants from lemon seedlings, the highest shoot number produced was observed by adding 2 mg/l BA. According to Carimi & De Pasquale (2003), the number of buds sprouted and shoots derived per explant, is influenced by the genotype, and this may explain the difference between *P. trifoliata* and *C. aurantium* recorded in our results. Therefore, by comparing the shoot number of six lemon varieties, only the three were affected by the BA concentration (Pérez-Tornero *et al.*, 2010). As refers to callus formation and on the contrary research of Molina *et al.* (2007), it appears that the addition of BA was not necessary for callus induction, but increased the percentage of callus formation on the explants.

3.2 Organogenesis from mature leaf explants

In some treatments on the etched points of the lower surface of leaves, callus formation was observed. Callus had different colour such as yellow, yellow - brown, brown and green (Table 2, Figure 1). The percentage of explants with callus is the average of two experiments. In *C. aurantium* explants, the highest callus formation was observed in the treatment 5.500 mg L^{-1} TDZ. The colour of callus was mostly yellow - brown. In *P. trifoliata* explants, the highest percentage of callus formation as observed in the treatments 2.0 mg L^{-1} NAA + 0.5 mg L^{-1} BA and 5.0 mg L^{-1} NAA + 0.5 mg L^{-1} BA, and the colour of callus was green. In Citrumelo explants, TDZ was the growth regulator which caused the maximum callus formation, alone or in combination with BA. Especially in the treatments 0.110, 1.100 and 3.300 mg L^{-1} TDZ and (0.110 TDZ + 0.5 BA), (1.100 TDZ + 0.5 BA), (3.300 TDZ + 0.5 BA) (mg L^{-1}), 100% of explants formed yellow or brown callus. High concentrations of BA did not increase callus formation. In Citrumelo explants, TDZ induced callus to a 100% in concentrations of 0.11, 1.1 and 3.3 mg L^{-1}, alone or in combination with BA.

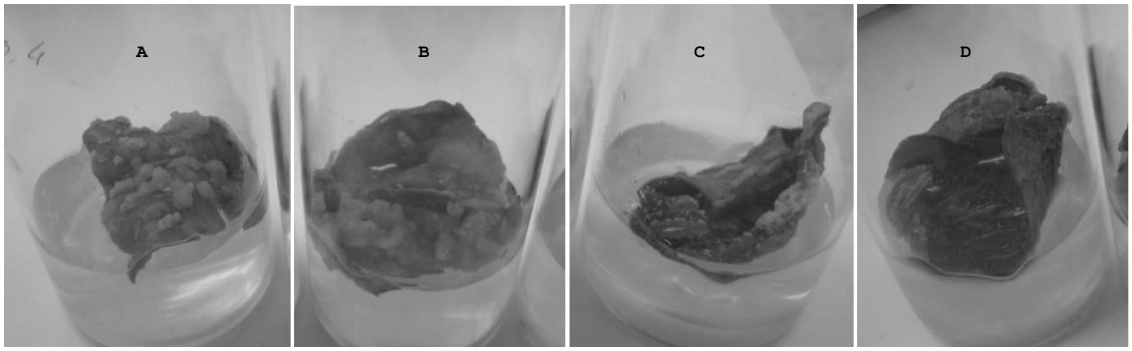

Figure 1. Callus formation on mature leaf explants of *C. aurantium*, on MS culture medium supplemented with various concentrations of TDZ and BA. A. 1.1 mg L^{-1} TDZ, yellow-brown callus. B. 5.5 mg L^{-1} TDZ, yellow-brown callus. C. 1.1 mg L^{-1} TDZ + 0.5 mg L^{-1} BA, yellow callus. D. 5.5 mg L^{-1} TDZ + 0.5 mg L^{-1} BA, brown callus.

Since callus formation is a stage of indirect organogenesis, callus formation is a positive result for proliferation, especially green colour callus (Tao *et al.*, 2002). In preliminary experiments by Esmaeilnia & Dehestani (2015) mature leaf explants were used, but were mostly died in a few days in culture and failed to produce adventitious shoots. However, Khan *et al.* (2009) reported successful regeneration of adventitious shoots from fully developed leaves form *in vitro* grown sweet orange seedlings.

Table 2. Callus formation on *C. aurantium* L., *P. trifoliata* L. and Swingle citrumelo mature leaf explants cultured *in vitro*. The effect of various concentrations of growth regulators on the percentage of callus formation per explant and callus colour.

Treatment (mg L^{-1})	*C. aurantium*		*P. trifoliata*		Swingle citrumelo
	Percentage of callus formation per explant	Callus colour	Percentage of callus formation per explant	Callus colour	Percentage of callus formation per explant
0	0	-	0	-	0
BA					
0.5	0	-	0	-	0
1.0	0	-	0	-	0
2.0	0	-	0	-	0
3.0	20	brown	0	-	0
4.0	0	-	0	-	0
NAA					
2.0	40	yellow-brown	0	-	0
5.0	0	-	33	green	0
8.0	0	-	0		0
NAA + BA					
2.0 + 0.5	33	brown	89	green	17
5.0 + 0.5	0	-	89	green	50
8.0 + 0.5	0	-	56	green	25
IAA + BA					
0.2 + 2.0	0	-	0	-	0
0.5 + 2.0	22	brown	0	-	0

0.2 + 3.0	17	brown	7	green	0
0.5 + 3.0	0	-	0	-	0
TDZ					
0.011	0	-	13	brown	60
0.110	14	yellow-brown	75	green	100
1.100	14	yellow-brown	50	brown	100
3.300	20	brown	11	brown	100
5.500	56	yellow-brown	18	brown	50
TDZ + BA					
0.011 + 0.5	38	brown	38	brown	33
0.110 + 0.5	17	yellow-brown	0	-	100
1.100 + 0.5	29	yellow	30	brown	100
3.300 + 0.5	0	-	13	brown	100
5.500 + 0.5	17	brown	0	-	0

The role of cytokinin in organogenesis has been studied in many plant species and is considered as absolutely essential for shoot formation (Molina *et al.*, 2007). However, callus formation is based on the type and concentration of growth regulator (Tao *et al.*, 2002). Thus, high concentrations of BA did not increase callus formation. This is probably attributed to the toxic effect of high BA concentrations, as it was previously reported for other citrus genotypes including *C. aurantium* (Molina *et al.*, 2007; Cervera *et al.*, 2008; Tallon *et al.*, 2013).

TDZ is a very active cytokinin and has given good results in proliferation and regeneration of several plants species (Thomas, 2003; Sanikhani *et al.*, 2006). In accordance to previous studies, it was found that the presence of TDZ was very efficient for callus formation in *C. aurantium* explants, alone or in combination with BA. In these cases, the colour of callus was mainly yellow - brown. On the contrary, in *P. trifoliata* the combination of NAA and BA in the culture substrate was more effective than TDZ for callus formation on leaf explants. It was confirmed that the ability of organogenesis is directly related to the genetic background of mother plants. This may depend on the different level of endogenous metabolites or hormones involved in the regeneration process (Khan *et al.*, 2009). Therefore, NAA led to green callus formation in *P. trifoliata*, just as in *Citrus grandis* leaf explants (Tao *et al.*, 2002), but not in *C. aurantium*. Research indicates that TDZ inhibits growth in plants formed from acquired buds, such as differentiation of roots, shoots and leaves. In some woody plants, TDZ concentrations less than 0.2 mg L^{-1} can be favorable or unfavorable for proliferation, depending on plant species (Mroginski *et al.*, 2004). In Citrumelo explants, we found that TDZ was the most appropriate growth regulator for inducing callus. Furthermore, as it concerns callus colour, it is reported that yellow or brown callus is not suitable for shoot induction, even on a suitable culture medium (Tao *et al.*, 2002).

The origin of the explant influences the age and the ability for organogenesis (Khan *et al.*, 2009). In our research, we focused on use of leaves from mature rootstock plants grown in the greenhouse for the following reasons; there is plenty of plant material available, many regenerating plants can be produced, and their commercial value can be estimated soon. The fact that we observed no shoot induction but only callus is perhaps due to the leaf age. Furthermore, callus formation at the base of explants in the *in vitro* cultivation of woody plants is attributed to the auxin that is accumulated at the base of the shoots (polar transport) in order to start cell proliferation, especially in the presence of cytokinins (Tao et al., 2002).

4. Conclusions

- Using mature explants of *P. trifoliata*, *C. aurantium* and Swingle citrumelo is a successful and quick way of *in vitro* propagation.
- The regeneration from mature leaves follows the indirect organogenesis, forming callus at the initial stage.
- The transfer of callus in a different culture medium is required, for shoot proliferation and rooting

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