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Short communication

Adventitious shoot regeneration from leaf thin cell layers in apple

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ABSTRACT

The present investigation was conducted to study organogenesis of a conventionally easy-to-regenerate ('Royal Gala') and a difficult-to-regenerate ('Freedom') apple cultivar using leaf transverse thin cell layers (tTCLs). The position of the leaf source, explant type and light regime applied to tTCLs were investigated as was their influence on the organogenic outcome. Light during the onset of regeneration delayed morphogenesis or, in 'Freedom', completely inhibited organogenesis on leaf tTCLs. Genotype and initial position of the leaf also influenced percentage regeneration and number of regenerated shoots per explant. This study demonstrates the successful application of leaf tTCLs for adventitious shoot regeneration in apple, even though further studies are needed to increase the number of shoots more than control explants. Shoot regeneration from apple leaf tTCLs serves as an alternative protocol for studying fundamental and applied aspects of regeneration.

1. Introduction

Adventitious shoot regeneration is a prerequisite for successful application of different biotechnological methods in apple breeding (Korban and Chen, 1992; Ou et al., 2008). Regeneration via organogenesis from leaf explants has been described for different apple rootstocks and scions (reviewed by Magyar-Tábori et al., 2010; Dobránszki and Teixeira da Silva, 2010). However, the use of thin cell layers (TCLs) as an explant has never been tested for any *Malus* species.

The TCL system is a simple yet successful regeneration technique in micropropagation and genetic transformation. Besides, it is a useful tool for studying different physiological and genetic pathways of plant development, and has been applied to the micropropagation of many ornamental plants, including model plant *Antirrhinum majus* (reviewed by Teixeira da Silva et al., 2007). It has been successfully used for the micropropagation of several difficult-to-propagate or recalcitrant woody plants, such as bamboo, cassava, pine or poplar (Nhut et al., 2003a). Amongst the woody fruits TCL technology has been developed for citrus, coconut palm, mangosteen (Fiore et al., 2002; Nhut et al., 2003b), and trifoliate orange (Van Le et al., 1999). Based on its success with other fruit species, we hypothesized that TCLs might also be successfully employed in apple organogenesis. From previous studies using conventional leaf and stem explants (Dobránszki et al., 2005; reviewed in detail in Dobránszki and Teixeira da Silva, 2010; Magyar-Tábori et al., 2010), 'Freedom' is considered to be a difficult-to regenerate cultivar while 'Royal Gala' is an easy-to-regenerate cultivar. The present study was thus undertaken to study the responsiveness of both cultivars to regeneration from leaf transverse TCLs (tTCLs) to assess whether these explants can be used to induce adventitious shoot regeneration in apple as well as or better than conventional protocols.

2. Materials and methods

In vitro apple shoots of 'Royal Gala' and 'Freedom' were pretreated for 3 weeks: 3-week-old shoots having 5–7 leaves about 35–40 mm in length were placed on shoot proliferation medium containing MS (Murashige and Skoog, 1962) basal medium supplemented with 100 mg l^{-1} *myo*-inositol, 0.7% agar-agar (plant cell cultured tested, SIGMA), 3% sucrose, 0.3 mg l^{-1} indole-3-butyric acid (IBA) and 0.2 mg l^{-1} gibberellic acid (GA_3) while the cytokinin concentration was adjusted to the optimal for each cultivar according to our earlier findings: 1.0 mg l^{-1} N^6-meta-hydroxy-benzyladenin (TOP) for 'Royal Gala' and 1.5 mg l^{-1} TOP for 'Freedom' (Dobránszki et al., 2005; Magyar-Tábori et al., 2010). The upper two, fully expanded young leaves of 3-week-old *in vitro* shoots were used as the explant source for regeneration experiments. The effects of the position of source leaves, the explant type and the light regime applied to leaf tTCL explants were studied, as follows (Table 1).

An experiment was conducted to test the appropriate donor position of leaves: either the 1st or 2nd terminal leaf. After removing the petiole and apex of leaves two types of explants were

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Table 1
Influence of the position of leaf explant source, explant type and light on adventitious shoot regeneration from tTCLs of leaves of different apple cultivars after 7 weeks of regeneration.[+]

No.	Treatments			'Royal Gala'		'Freedom'	
	Position of source leaf	Explant type	Light regime[a]	Regeneration per cent	No. of shoots/explant	Regeneration per cent	No. of shoots/explant
1	1st leaf	Control	D + L	100 a	5.6 b	39 a	2.1 a
2		tTCL	D + L	97 a	3.2 c	34.5 a	1.5 a
3			L	92 a	3.2 c	0 d	0 b
4	2nd leaf	Control	D + L	100 a	8.0 a	25 b	2.1 a
5		tTCL	D + L	78 b	5.1 b	11 c	1.4 a
6			L	71 b	2.7 c	0 d	0 b

[a] D + L: explants were cultivated in the dark at the onset of the regeneration process for 3 weeks, L: explants were cultured in light throughout the regeneration process.
[+] Means followed by the same letter within a column are not significantly different at $P < 0.05$ according to Tukey's test. Data of each apple cultivar was assessed separately.

prepared: (1) control (i.e., according to Dobránszki et al.'s (2005) optimized protocol) explants, in which leaves were cut transversely into two strips about 5 mm wide, and (2) tTCLs 0.1–0.3 mm thick. All explants were cut in a sterile solution of citric acid (0.15 g l^{-1}) and ascorbic acid (0.1 g l^{-1}) to avoid tissue browning and death during cutting (Dobránszki and Teixeira da Silva, 2010). Leaf explants were placed into polystyrene Petri dishes (92 mm in diameter, 25 ml of medium) adaxial side down onto regeneration media, which consisted of MS salts, B_5 vitamins supplemented with *myo*-inositol (100 mg l^{-1}), gelrite (0.25%), sucrose (3%), α-naphthalene acetic acid (NAA, 0.2 mg l^{-1}) and thidiazuron (TDZ), whose concentration was adjusted to the optimal for each cultivar according to our earlier findings: 0.5 mg l^{-1} for 'Royal Gala' (Dobránszki et al., 2005) and 5.0 mg l^{-1} for 'Freedom' (Magyar-Tábori et al., 2010). Control explants and half of the tTCL explants were cultured in the dark at 24.5 °C for 3 weeks prior to placing them in the light at 22 °C at a 16-h photoperiod (white heat fluorescent lamps, 400–700 nm). Light intensity was increased weekly: it was 35 μmol s^{-1} m^{-2} during the first week, 70 μmol s^{-1} m^{-2} during the second week and 105 μmol s^{-1} m^{-2} from the third week (designated the D + L light regime). There was no dark incubation period at the beginning of regeneration in the case of the other half of the tTCL explants. At the onset of regeneration they were cultured in light under low light intensity (35 μmol s^{-1} m^{-2}) for four weeks prior to increasing the light intensity as in the other treatments (designated the L light regime).

Cultures were observed weekly to score responsive explants showing callus formation or bud/shoot regeneration. After 7 weeks of culture the percentage of explants that regenerated shoots (regeneration per cent, *R*%) and the number of regenerated shoots per explant (SN) were recorded. Six control explants and 12 tTCL explants were placed separately per Petri dish and 10 Petri dishes were used for each treatment; i.e., 60 + 60 explants were analysed in control treatments and 120 explants were evaluated in each tTCL treatment. The data were analysed by analysis of variance followed by Tukey's test (at $P < 0.05$) by using SPSS 13.0 for Windows software.

3. Results and discussion

Callus appeared after the first week of culture of 'Royal Gala' although the percentage differed depending on the treatment. Callus formed on average 50–62% of control explants but on 72–93% of tTCL explants cultured in the dark (values based on average percentages for both 1st and 2nd leaves); lower values were always recorded on explants originating from the 2nd leaf. When tTCL explants were cultured in the light, callus formation percentage varied between 15 and 17%. Almost every explant (95–100%) formed callus by the end of the second week except for tTCLs originating from the first leaf cultured in light (88%), although 100% of tTCLs formed callus by the end of the third week of culture.

In the first week, only 8–33% of control 'Freedom' explants formed callus. By the second week; however, 100% of control explants and tTCLs cultured in the dark formed callus. Culturing tTCLs in light delayed callus formation. The percentage of responsive TCLs was only 10–22% after the second week but reached 100% after 4 or 5 weeks of culture and it was always higher on explants originating from the 2nd leaf.

Adventitious shoot buds were initiated within 4 weeks in each treatment for 'Royal Gala'. 70–82% of control explants, 30–52% of tTCLs cultured in the dark and 35% of tTCLs cultured in the light developed buds at the end of the 4th week. Bud development began at the same time in 'Freedom', although the percentage of responsive explants was much lower than for 'Royal Gala'. When explants were cultured in the dark during the first 3 weeks (D + L light regime), 5–7% of control explants and 3–10% of tTCLs showed bud differentiation by the 4th week. No bud development could be observed on tTCLs cultured in the light (L light regime) by the end of the experiments.

The regeneration percentage (*R*%) and the number of regenerated shoots per explant (SN) after 7 weeks of regeneration in the different treatments and for both cultivars are presented in Table 1. *R*% for 'Royal Gala' decreased significantly to 71–78% when tTCLs originated from the 2nd leaf but in the other treatments it was high (92–100%). SN was always highest when control explants were used; however, there were no significant differences in the SN between control explants originating from the 1st leaf (5.6) and tTCLs cultured in the dark having originated from the 2nd leaf (5.1). When 2nd leaves were the source of explants, dark treatment (D + L light regime) approximately doubled SN (2.7 vs. 5.1) (Fig. 1).

Shoot regeneration ability of 'Freedom' was much lower than for 'Royal Gala'. No shoots regenerated when tTCLs were cultured in the light (L light regime). *R*% decreased significantly when 2nd leaves of shoots were used for regeneration (from 39% to 25% in control explants and from 34.5% to 11% in tTCLs) and the percentage of responsive explants was significantly lower using tTCLs compared to control explants (11% vs. 25%) when 2nd leaves were the source of explants. SN showed a decreasing tendency when tTCLs were used, although differences detected between explant types were not significant. Neither were significant differences observed in SN between the position of source leaves, i.e. 1st vs. 2nd leaf (Fig. 1).

Our results show that light delayed callus formation in both cultivars but this effect was much clearer in the difficult-to-regenerate cultivar ('Freedom'). Shoot regeneration ability was also strongly influenced by genotype. In easy-to-regenerate cultivar ('Royal Gala') there were no differences between the light regimes, i.e. D + L vs. L treatments; light during the onset of regeneration did not affect the regeneration percentage. On the contrary, in the difficult-to-regenerate cultivar light completely inhibited shoot development on tTCLs. These delaying and/or inhibiting effects of light at the begin of organogenesis from apple leaf tTCLs correspond to earlier findings on positive effects of darkness on regeneration from