POLISH SOCIETY FOR HORTICULTURAL SCIENCE

Folia Hort. 27/2 (2015): 183-190

DOI: 10.1515/fhort-2015-0029

FOLIA HORTICULTURAE

Published by the Polish Society for Horticultural Science since 1989

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Plant thin cell layers: update and perspectives

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ABSTRACT

Thin cell layers (TCLs) are small and versatile explants for the *in vitro* culture of plants. At face value, their morphogenic productivity may appear to be less than conventional explants, but once the plant growth correction factor and geometric factor have been applied, the true (potential) productivity exceeds that of a conventional explant. It is for this reason that for almost 45 years, TCLs have been applied to the in vitro culture of almost 90 species or hybrids, mainly ornamentals and orchids, but also to field and vegetable crops and medicinal plants. Focusing on 12 new studies that have emerged in the recent past (2013-2015), this paper brings promise to other horticultural species that could benefit from the use of TCLs.

Key words: model plants, organogenesis, plant biotechnology, TCLs

THIN CELL LAYERS: HISTORY AND APPLICATIONS

The explant is one of the key factors in the success of a plant tissue culture protocol. A number of biotic factors including the genotype (species and cultivar), the tissue or organ from which it is prepared, the age of mother tissue or organ, its size and its shape are all united to result in the success, or failure, of *in vitro* morphogenesis.

The concept of the thin cell layer (TCL) system originated from Tran Than Van (1973), who described that *in vitro* reprogramming and thereby regeneration of an organ or embryo might be possible by isolation of one or a few (3-6) layers of differentiated cells from any organ or tissue. TCLs represent a multicellular system, they are explants of small sizes (in general < 1-2 mm in thickness prepared from differentiated plant organs, and they have some inherent temporal and spatial organization (Tran Than Van 2003).

The shape as well as the size of TCLs as explants depends both on the organ from which they are derived and the way in which they are prepared, i.e. if they are longitudinal (lTCL) or transversal (tTCL) (Tran Than Van 2003). The actual regeneration capacity of TCL explants is often much higher than thicker conventional explants partly due to having a higher ratio of morphogenic cells and better transport between the medium and these cells (Tran Than Van 2003). Two new concepts, the plant growth correction factor (GCF) and the geometric factor (GF) (Teixeira da Silva and Dobránszki 2011, 2014) demonstrated the extraordinary productivity of TCLs compared to conventionally sized explants, even in cases when the actual or observed productivity of TCLs seems to be lower.

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Over 40 years since the birth of the TCL concept (Tran Than Van 1973) morphogenesis in 77 different plant species or hybrids, including many horticultural species, were successfully reported via using TCLs as the explant (Tab. 1), based on the reviews of Teixeira da Silva and Dobránszki (2013) and Teixeira da Silva (2013). These studies focused primarily on ornamental plants (about 50% of species), mainly on different orchid species and hybrids but little attention was paid to field or vegetable crops (25% of the studied species) and even less to medicinal plants (only 8% of the studied species). Since those reviews, 12 new studies in 10 new species/plants have emerged, and these studies are the focus of this review.

TCLs: RECENT TRENDS AND ACHIEVEMENTS

Recently published studies on *in vitro* regeneration from TCLs are summarized in Table 2. The mass propagation of orchids remains important, mainly if new cultivars or hybrids need to be introduced to the market. It is thus not surprising that *in vitro* propagation protocols using TCLs have been published on more orchid cultivars/hybrids, namely *Brasilidium forbesii* (Hook.) Campacci (Gomes et al. 2015), *Cymbidium* Twilight Moon 'Day Light' (Teixeira da Silva 2014), and *Dendrobium aqueum* (Lindley) (Parthibhan et al. (unpublished)) (Tab. 2).

TCLs have brought a focus to several endangered medicinal plants. *Psychotria ipecacuanha* (Brot.) Stokes, a medicinal plant, contains a drug with medicinal importance and its main bioactive compounds are the isoquinoline alkaloids, emetine and cephaeline, it has expectorant and anti-vomiting effects, it is an amoebicide, and is endangered due to large-scale harvesting of wild plants (Souza et al. 2008). *In vitro* regeneration through organogenesis and somatic embryogenesis was achieved by using stem and leaf tTCLs (Tab. 2). Bulbous ceropegia (*Ceropegia bulbosa* Roxb.) is an endangered plant with both medicinal and food values (Teixeira da Silva 2015). TCL system for direct or indirect organogenesis served as a feasible and reliable mass propagation protocol for bulbous ceropegia (95% of TCLs were responsive with 22.2 shoots/tTCL; Tab. 2). *Paris polyphylla* Sm. is a traditional virtuous plant whose rhizome is used because of its high saponin content. Regeneration of *P. polyphylla* mini-rhizomes on tTCLs (Raomai et al. 2015; Tab. 2) is a suitable method for mass propagation, germplasm conservation and also the production of secondary metabolites. Somatic embryogenesis (141 somatic embryos/tTCL, 88.3% of tTCLs were responsive) was induced on tTCLs from microrhizomes of another herb and medicinal plant, ginger (*Zingiber officinale* Roscoe) (Thingbaijam and Huidrom 2014; Tab. 2).

Agave fourcroydes Lem. has multiple functions: its fiber is used industrially, it is the source of some steroidal precursors and cellulose and biofuels (Monja-Mio and Robert 2013). Direct somatic embryogenesis on stem TCLs with a mean productivity of 92.22 somatic embryos (SEs)/explant (Monja-Mio and Robert 2013; Tab. 2) reached or exceeded the efficacy of previously reported studies on indirect embryogenesis in other *Agave* species; 44-46 SEs/explant excised from cotyledons, shoot tips or *in vitro* leaves of *Agave vera-cruz* Mill. (Tejavathi et al. 2007) or on average 0.21-98.5 SEs/explant depending on the genotype with a maximum of 139 SEs/explant in a selected genotype when explants originated from young leaf blades of *Agave tequilana* Weber cv. 'Azul' (Portillo et al. 2007).

Stevia rebaudiana Bertoni is important in the food industry, as a healthy alternative to artificial sweetener (Meireles et al. 2006). Micropropagation of *Stevia rebaudiana* by using tTCLs from seedling hypocotyls (Ramirez-Mosqueda and Iglesisas-Andreau 2015) was more efficient (31.16 shoots/tTCL explant; Tab. 2) than previously described methods using conventional explants (9.56 and 22.5 shoots on nodal segment explants, respectively (Modi et al. 2012, Nower 2014), allowing the commercial-scale production of its propagation material.

More than mass propagation protocols, TCLs were used for studying the developmental processes of adventitious rooting and xylogenesis in *Arabidopsis thaliana* (Della Rovere et al. 2013, 2015) (Tab. 2), a model crop species that has already employed TCLs for regeneration (Tab. 1). TCLs were important explants in the regeneration of genetically transformed pyrethrum (*Tanacetum cinenariifolium* (Trev.) Sch. Bip.) carrying the green fluorescent protein (GFP) gene (Mao et al. 2014). Pyrethrum is well-known industrial crop and the source of the insecticide pyrethrin. Pyrethrum used to be a recalcitrant species in tissue culture, and only a few studies had described successful shoot regeneration. Obukosia et al. (2005) induced organogenesis on callus with a productivity of 4-5 shoots/callus after an 8-week-long culture period. Direct shoot organogenesis was induced on pyrethrum petiole and leaf segments in 18% and 70% of explants, respectively (Hedayat et al.

Table 1. Different plant species where TCL explants was successfully used for *in vitro* organogenesis or embryogenesis (based on the reviews of Teixeira da Silva and Dobránszki 2013, and Teixeira da Silva 2013)

Plant groups	Species/hybrids
Model plants	*Arabidopsis thaliana* (L.) Heynh. Snapdragon (*Antirrhinum majus* L.) Tobacco (*Nicotiana tabacum* L.)
Orchids	*Aerides maculosum* *Aranda* Deborah *Coelogyne cristata* Lindl. *Cymbidium aloifolium* (L.) Sw. *Cymbidium bicolor* *Cymbidium* cv. 'Sleeping Nymph' hybrid *Cymbidium* cv. Twilight Mon 'Daylight' hybrid *Dendrobium candidum* Wall ex Lindl. *Dendrobium draconis* *Dendrobium gratiosissimum* *Dendrobium nobile* Lindl. *Doritaenopsis* (*Doritis* × *Phalaenopsis*) New candy × *D.* (Mary Anes × Ever Spring) hybrid *Eria dalzelli* *Liparis elliptica* *Paphiopedilum* Deperle és *P. Armeni* White *Renanthera* Tom Thumb 'Qilin' *Rhynchostylis gigantea* *Spathoglottis plicata* *Xenikophyton smeeanum*
Other ornamentals	African violet (*Saintpaulia ionantha* H.Wendl.) Amaranth (*Amaranthus edulis* Speg.) Begonia (*Begonia rex* Putz.) Chrysanthemum (*Dendranthema grandiflora*) *Citrus* sp. Easter lily (*Lilium longiflorum* Thunb.) Gentian (*Gentiana* spp.) Geranium hybrid (*Pelargonium* × *hortorum*) Gerbera (*Gerbera jamesonii* Hooker f.) Gladiola (*Gladiolus* spp.) Gloxinia (*Sinningia speciosa* Baill.) Ornamental sunflower (*Helianthus annuus* L.) Parrot's flower (*Heliconia psittacorum* L.f.) Passion fruit (*Passiflora edulis* Sims.) Petunia (*Petunia hybrida*) Rose (*Rosa hybrida*) Sweet iris (*Iris pallida*) Tea (*Camellia sinensis* (L.) Kuntze) Wishbone flower (*Torenia fournieri* Lind.)
Field crops and vegetables	Common bean (*Phaseolus vulgaris* L.) Cowpea [*Vigna unguiculata* (L.) Walp] Pearl lupine (*Lupinus mutabilis* Sweet.) *Lycopersicon* species: *L. cheesmanii*, *L. chilense*, *L. chmielewskii*, *L. hirsutum*, *L. parviflorum*, *L. peruvianum*, *L. pimpinellifolium* Rapeseed (*Brassica napus* L.) Rice (*Oryza sativa* L.) Sesame (*Sesamum indicum* L.) Sorghum [*Sorghum bicolour* (L.) Moench] Spinach (*Spinacia oleracea* L.) Sugarcane (*Saccharum* spp. interspecifikus hibridek) Tomato (*Lycopersicon esculentum* L.) White lupine (*Lupinus albus* L.)
Medicinal plants	*Ceropegia* fajok Ceylon spinach [*Talinum triangulare* (Jacq.) Willd.] False daisy (*Eclipta alba* L.) Ginseng (*Panax* sp.) Jambu (*Acmella oleracea* L.) St. John's wort (*Hypericum perforatum* L.)
Woody plants	Lemon [*Citrus limon* (L.) Burm.] Sweet orange [*Citrus sinensis* (L.) Osb.] *Pinus* species: *P. kesiya*, *P. roxburghii*, *P. wallichiana*, *P. sylvestris*, *P. pinea*, *P. patula* and *P. pinaster* Oil palm (*Elaeis guineensis* Jacq.) Peach palm (*Bactris gasipaes* Kunth) Trifoliate orange (*Ponicirus trifoliata* L. Raf.)

Table 2. Use of thin cell layers (TCLs) in regeneration studies from 2013-2015 (chronological listing)

Genus, species, cultivar	Culture medium, PGRs, additives, other conditions*,**,***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	Reference
Arabidopsis thaliana§,‡	lTCLs (0.5 mm × 8 mm; 6/7 cell layers; seedling-derived) cultured in PGR-free MS + 1% sucrose or MS + 10 µM IBA + 0.1 µM Kin. lTCLs in continuous darkness for 22 d, then white light for 6 h, then darkness for 14 days. pH, PP and carbon source NR. 25 µmol m^{-2} s^{-1}. 24 ± 2°C. 70 ± 5% RH.	lTCLs used, relative to *in planta* cells, to assess the induction of adventitious root meristemoids from wild-type and mutant strains of *A. thaliana*. The involvement of specific genes and proteins in adventitious root development and xylogenesis was clarified. Acclimatization not performed.	Della Rovere et al. 2013, 2015
Agave fourcroydes Lem.	tTCLs (0.5-1.0 mm thick) from base of leaves and stems of 5-7 cm tall *in vitro* plantlets. MS + L2 vitamins (Phillips and Collins 1979) + 2.26 µM dicamba or 2.07 µM picloram + 3% sucrose + 0.3% agar + 0.3% Phytagel™ (SEIM). PGR-free ½MS + 3% sucrose + 0.3% agar + 0.3% Phytagel™ (SEGM). pH 5.75. Darkness (SE induction) or 16-h PP + 45 µmol m^{-2} s^{-1} (SE germination phase). 25 ± 2°C.	In the presence of dicamba, 92.22 SEs/stem tTCL formed (81.72 SEs/stem tTCL with picloram). Apical tissues formed more SEs than basal tissue. Embryogenic response of 13 clonal lines varied widely (0-117 SEs/stem tTCL) after exposure to dicamba. Vascular procambium cells showed maximum competence towards SE formation. 85% survival of acclimatized plantlets in 3:1 vermiculite + soil.	Monja-Mio and Robert 2013
Ceropegia bulbosa Roxb.	Surface-disinfected tTCLs (1-4 mm thick) from nodal segments of *ex vitro* plants. MS + 4.5 µM 2,4-D + 2.2 µM BA (CIM). MS + 8.8 µM BA + 0.27 µM NAA (SIM). PGR-free ½MS (RIM). pH 5.6. 3% sucrose. 0.8% agar. 16-h PP. PPFD NR. 25 ± 2°C. 50 ± 5% RH.	95% of tTCLs formed callus in CIM. 22.2 shoots/tTCL on SIM. 89% of acclimatized plantlets survived in garden soil. No variation among tTCL-derived plantlets assessed by ISSR.	Dhir and Shekhawat 2014
Tanacetum cinenariifolium (Trev.) Sch. Bip.	tTCLs (0.5-1 mm thick) from internodes of seedling stems grown on MS medium + 3% sucrose. tTCLs cultured on MS medium + 0.7% agar + 3% sucrose + 9 combination of BA (2, 4,8 mg/l) and IBA (0.2, 0.4, and 0.8 mg/l). pH 5.8. 14-h PP, 3000 lux, 25°C.	Regeneration percentage was 31% after one week of culture. By the 3rd week, 42% of tTCLs regenerated and developed an average of 2 shoots/tTCL.	Mao et al. 2014
Cymbidium Twilight Moon 'Day Light'	tTCLs (1-2 mm thick) from PLBs. TC + 1/2 mg/l 2,4-D or 1 mg/l TDZ (CIM). TC + 1 mg/l AdS or 1/2/4 mg/l BA or 1/2 mg/l Kin or 1 mg/l ZR or 1 mg/l 2iP (PIM). 2 mg/l tryptone. pH 5.3. 2% sucrose. 0.8% Bacto agar. 16-h PP. 45 µmol m^{-2} s^{-1}. 25°C.	26 PGRs tested. Light favored PLB formation more than darkness. Controls formed 8.3 new PLBs/tTCL but all PIM options resulted in a maximum of 1.1 new PLBs/tTCL (however, these values would need correction by the Plant Growth Correction Factor; Teixeira da Silva and, Dobránszki 2014). Acclimatization not performed.	Teixeira da Silva 2014
Zingiber officinale var. 'Nadia'	tTCLs (0.5 mm thick) of *in vitro*-derived microrhizome. MS + 2 mg/l BA + 1 mg/l NAA (MIM). MS + 2 mg/l 2,4-D (CIM). MS + 0.2 mg/l 2,4-D + 6 mg/l BA (SEIM). pH 5.8. 3% (CIM, SEIM) or 8% (MIM) sucrose. 0.8% agar. 16-h PP. 25 µmol m^{-2} s^{-1}. 25 ± 2°C.	88.3% of tTCLs formed callus on CIM. 141 SEs/tTCL formed on SEIM. Acclimatization claimed, but not quantified.	Thingbaijam and Huidrom 2014
Psychotria ipecacuanha (Brot.) Stokes	tTCLs (0.5-1.0 mm thick) of *in vitro*-derived stems (after 5 subcultures). MS + 3 mg/l IBA + 0.3 mg/l TDZ (SIM). MS + 1 mg/l IBA + 0.3 mg/l TDZ (SEIM). 100 mg/l *myo*-inositol. pH 5.8. 3% sucrose. 0.28% Gellan gum®. 16-h PP. 20-30 µmol m^{-2} s^{-1}. 25 ± 2°C.	9.1% of tTCLs formed SEs on SEIM. 22% of tTCLs formed shoots on SIM. Red, blue and white LEDs stimulated shoot formation from tTCLs but green LEDs inhibited shoot formation. Acclimatization not performed.	Botero Giraldo et al. 2015¶
Brasilidium forbesii (Hook.) Campacci	tTCLs (1 mm thick) and lTCLs (size NR) from 2 mm wide protocorms. WPM + 1 µM BA (PIM). WPM + 3 g/l AC (SIM, RIM).	lTCLs formed PLBs better than tTCLs: 24.6 new PLBs/lTCL vs 21.3 new PLBs/tTCL. 100% survival of plantlets when acclimatized in vermiculite.	Gomes et al. 2015

Table 2. Use of thin cell layers (TCLs) in regeneration studies from 2013-2015 (chronological listing), continued

Genus, species, cultivar	Culture medium, PGRs, additives, other conditions*,**,***	Remarks, experimental outcome and maximum productivity, acclimatization and variation	Reference
Stevia rebaudiana Bertoni	tTCLs (0.3-0.5 mm thick) from hypocotyls of seedlings (age NR). MS + 6.78 µM 2,4-D (SIM). PGR-free ½MS (RIM). 0.22% Phytagel® (SEGM). pH 5.8. 3% sucrose. 16-h PP. 50 µmol m^{-2} s^{-1}. 25 ± 2°C.	31.16 shoots/tTCL in SIM. 100% of shoots rooted on RIM and 90% of acclimatized plantlets (10-15 cm) survived in peat moss™ + agrolite™ (1:1).	Ramirez-Mosqueda and Iglesisas-Andreau 2015
Paris polyphylla Sm.	tTCLs (0.5 mm thick) from basal part of microrhizomes. ½MS + 0.5 mg/l BA (microrhizome induction medium). PGR-free ½MS (SIM). pH 5.8. 3% sucrose. 0.8% agar. Darkness (microrhizome induction). 14-h PP + 60 µmol m^{-2} s^{-1} (SIM). 25 ± 2°C.	86.6% of tTCLs formed microrhizomes. >95% survival of acclimatized plantlets (conditions NR). tTCL-derived rhizomes had 1.4-fold higher steroidal saponins than field-derived rhizomes. Acclimatization not performed.	Raomai et al. 2015
Dendrobium aqueum Lindley	tTCLs (0.5 mm thick) from stem of *in vitro* shoots. ½MS + 1.5 mg/l 2iP (SEIM). pH 5.8. 2% sucrose. 0.7% agar. 16-h PP + 35 µmol m^{-2} s^{-1}. 23 ± 2°C.	42.66 globular SEs/tTCL in 10.33% of explants. 2iP-free SEIM + 0.5 mg/L ZR induced EC in 41.42% of tTCLs. SEIM + 0.5 mg/l BA formed 34 SEs/tTCL on 14.7% of tTCLs. 2iP-free SEIM + 1 mg/L IBA (SEIM2) induced EC in 52.33% of tTCLs (7.4 SEs/tTCL). SEIM2 + 0.5 mg/l PVP formed 24 SEs/tTCL on 19.89% of tTCLs. Acclimatization not performed.	Parthibhan et al. 2015 (unpublished)

2,4-D, 2,4-dichlorophenoxyacetic acid; 2iP, N^6-(2-isopentenyl) adenine; AC, activated charcoal; AdS, adenine sulphate; BA, N^6-benzyladenine (BA is used throughout even though BAP (6-benzylamino purine) may have been used in the original, according to Teixeira da Silva (2012a); CIM, callus induction medium; EC, embryogenic callus; IBA, indole-3-butyric acid; ISSR, inter simple sequence repeat; Kin, kinetin (6-furfuryl aminopurine); LED, light-emitting diode; lTCL, longitudinal TCL; MIM, microrhizome induction medium; MS, Murashige and Skoog (1962) medium; NAA, α-naphthaleneacetic acid; NR, not reported in the study; PIM, PLB induction medium; PLB, protocorm-like body; PGR, plant growth regulator; PP, photoperiod; PPFD, photosnthetic photon flux density; PVP, polyvinylpyrrolidone; RH, relative humidity; RIM, root induction medium; SE, somatic embryo; SEGM, somatic embryo germination medium; SEIM, somatic embryo induction medium; SIM, shoot induction medium; TC, Teixeira *Cymbidium* medium (Teixeira da Silva 2012c); TDZ, thidiazuron (*N*-phenyl-*N'*-1,2,3-thiadiazol-5-ylurea); tTCL, transverse TCL; WPM, woody plant medium (Lloyd and McCown 1980); ZR, zeatin riboside. * Even though calli was used in the original, the term callus has been used here based on recommendation of Teixeira da Silva 2012b. ** The original light intensity reported in each study has been represented since the conversion of lux to µmol m^{-2} s^{-1} is different for different illumination (main ones represented): for fluorescent lamps, 1 µmol m^{-2} s^{-1} = 80 lux; the sun, 1 µmol m^{-2} s^{-1} = 55.6 lux; high voltage sodium lamp, 1 µmol m^{-2} s^{-1} = 71.4 lux (Thimijan and Heins 1983). *** All percentage values = w/v basis. § Columbia (Col) and Wassilewskija (Ws) ecotypes, of *QC25::GUS*, *DR5::GUS*, *PIN1::GUS*, *LAX3::GUS*, *pAGL42::GFP* and *pWOX5::GFP* lines (all in the Col background) and of the *sur2-1* mutant (Ws background) (Della Rovere et al. 2013). ‡ Homozygous *scr-1* and *shr-1* [Wassilewskija (Ws) wild type (wt)] and *lax3*, *aux1-21* and *lax3/aux1-21* [Columbia (Col) wt] null mutants, and of *pSCR::GFP* (Ws background), *AUX1::GUS*, *LAX3::GUS* and *DR5::GUS* lines (Col background) (Della Rovere et al. 2015). ¶ Claims of somatic embryogenesis without sufficient, or with unconvincing, proof (cytological, histological, genetic), i.e., only photos of macromorphology

2009). In the Mao et al. (2014) study, stem-derived tTCLs were induced to form shoots and all putative transformant lines regenerated from TCLs were GFP-positive.

CONCLUSIONS AND FUTURE PERSPECTIVES

The most obvious future expansion of the use of TCLs is for establishing a regeneration system for a plant that has only displayed a weak, or no, regeneration potential *in vitro*. A second possibility is to use this sensitive cellular/tissue system for medicinally, commercially or ornamentally important plants for which no *in vitro* regeneration system exists. A third possibility for better exploiting the potential of TCL as a regeneration system is to utilize explants of different origin, i.e. from organ or tissue types that have absolutely not yet been used or that were used for organogenesis or embryogenesis in a given species but only displayed low efficacy. This possibility was tested for apple. There are only few reports on shoot regeneration from stem segments in apple, and the productivity of stem segments was lower than that of leaf segments. Shoot regeneration was not obtained in the stem segments of cultivars 'Åkerö', 'McIntosh', and 'Gravenstein', and 'M.26' rootstock. In 'McIntosh Wijcik', shoots regenerated on stem segments with a 5% regeneration percentage while the number of regenerated shoots was between 6.4 and 16.3 on leaf explants, depending on the cultivar (Welander 1988). Successful shoot regeneration was achieved by some authors using internodal stem segments in some apple cultivars (in 'Golden Delicious', 2.5 shoots/stem explant with a regeneration frequency of 22.9%; in 'Royal Gala', 17-93% regeneration frequency and in 'Gale Gala', 19.5 shoots/whole stem with a regeneration frequency of 36.8%) but generally with low frequency (Belaizi et al. 1991, Liu et al. 1998, Bommineni et al. 2001).

To test this third possibility, tTCLs (0.2-0.3 mm thick) were prepared form the stem between the first and second leaves of *in vitro* apple shoots of cultivars 'Royal Gala' and 'Freedom'. Shoot regeneration was successful after a 7-week regeneration period in both cultivars (Fig. 1). In 'Royal Gala', which is an easy-to-regenerate cultivar, the regeneration percentage of stem tTCLs was significantly highest if tTCLs were cultured under light (L) and tended to be better if cultured in an inverse orientation (in a normal orientation 6.25% and 37%, in an

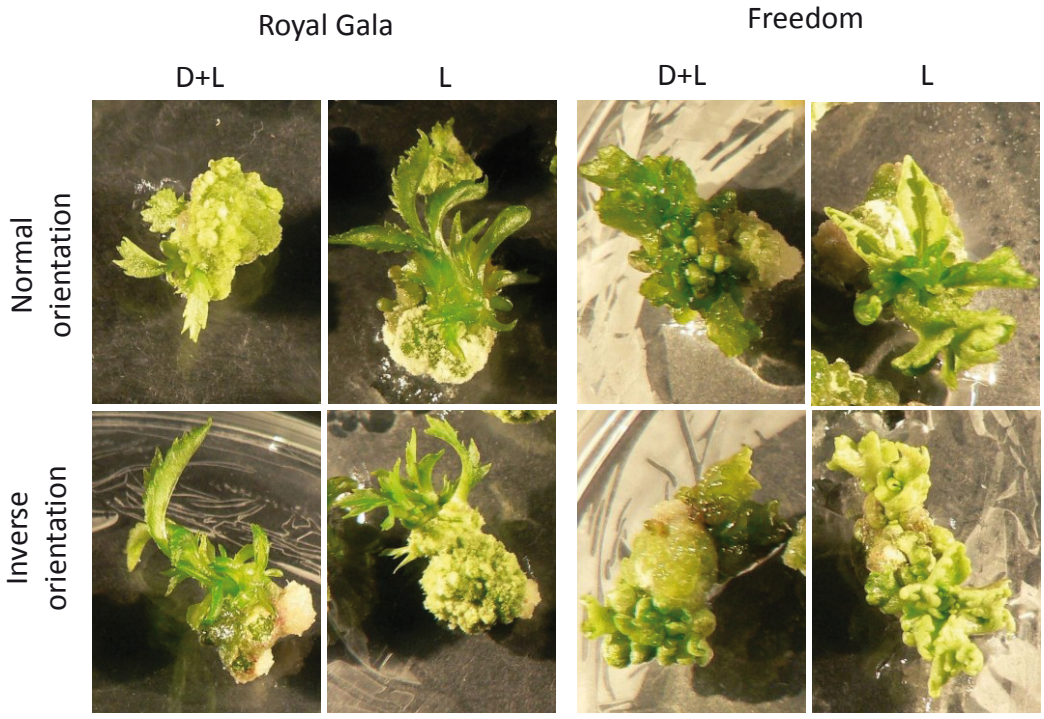

Figure 1. Adventitious shoot regeneration from apple (*Malus* × *domestica* cultivars 'Royal Gala' and 'Freedom') used to derive stem transverse thin cell layers (tTCLs; 0.2-0.3 mm thick) after 7 weeks in culture on regeneration medium (Dobránszki and Teixeira da Silva 2011) supplemented with 22.7 μM thidiazuron and 1.07 μM α-naphthaleneacetic acid. D+L: culture of explants for 3 weeks in the dark and then in the light for 4 weeks; L: culture of explants in the light for 7 weeks (growth conditions were the same as those described for leaf tTCLs in Dobránszki and Teixeira da Silva 2011). Explants were placed on medium either in a normal orientation of the stem or in an inverse orientation

inverse orientation 16% and 43% in D+L and L, respectively). However, regeneration capacity (43%, 2.2 shoots/stem tTCL) was below that of leaf tTCLs (78%, 5.1 shoots/leaf tTCL; Dobránszki and Teixeira da Silva 2011). In the difficult-to-regenerate cultivar, the regeneration capacity of 'Freedom' was the same as on leaf tTCLs (34.5%, 1.5 shoots/leaf tTCL; Dobránszki and Teixeira da Silva 2011) when stem tTCLs were cultured in an inverse orientation under L (43%, 2.1 shoots/stem tTCL). While shoot formation was completely inhibited on 'Freedom' leaf tTCLs, it was stimulated on stem tTCLs of this cultivar, the percentage of regenerating explants was double under L (i.e., 43%) than when a 3-week-long D+L period was inserted at the beginning of regeneration (i.e., 21%).

A forth possibility is to apply TCLs for studying the hormonal and genetic/molecular mechanisms underlying tissue differentiation, as has already been successfully used for roots in *A. thaliana* (Della Rovere et al. 2013, 2015).

FUNDING

The authors did not receive funding for this research.

AUTHOR CONTRIBUTIONS

Both authors contributed to all aspects of this manuscript, including development of the ideas, writing, revisions and joint responsibility for the content.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Received November 12, 2015; accepted December 2, 2015