J Plant Growth Regul (2014) 33:881–895
DOI 10.1007/s00344-014-9437-x

Dissecting the Concept of the Thin Cell Layer: Theoretical Basis and Practical Application of the Plant Growth Correction Factor to Apple, *Cymbidium* and Chrysanthemum

Jaime A. Teixeira da Silva · Judit Dobránszki

Received: 23 December 2013 / Accepted: 15 May 2014 / Published online: 22 June 2014

Abstract A thin cell layer (TCL) is a thin layer of plant cells. TCLs have served as a simple, but important biotechnological tool in plant science, with several dozen crop species having had tissue culture regeneration protocols developed using TCLs generated from multiple explant sources. There are two types of TCLs, transverse TCLs, or tTCLs and longitudinal TCLs, or lTCLs. The former is the most common, ranging from 100 μm to 1–2 mm in thickness, usually cutting through several tissue types. In contrast, the latter usually targets a very specific layer of cells or tissues, and may vary in length but is as thick as a tTCL. The developmental question that needs to be addressed will determine the choice between one or the other and its use in plant tissue culture. The often unappreciated beauty of the TCL is not so much in its actual regeneration capacity, but rather in its potential regeneration capacity. Herein, we use data from three model species, a woody temperate fruit tree, *Malus* sp. (apple; Rosaceae), and two herbaceous ornamentals, *Cymbidium* (orchid; Orchidaceae) and *Dendranthema* (chrysanthemum; Asteraceae), to demonstrate the theory and functionality of TCLs. Moreover, using a new concept, the plant growth correction factor, or GCF, the ability to theoretically predicts the organogenic outcome in vitro is presented through mathematical models based on the geometric analysis of explant size and shape. A new factor, the geometric factor, or GF, was also determined for all three plants to compare regeneration from different explant types with different shapes. The GF, which is calculated, is independent of plant species or any in vitro conditions, but depends only on the size and shape of the explant and on tissue that is capable of regeneration. The GF and GCF would, in theory, allow for the direct comparison of plant in vitro studies in different laboratories provided that explant size is known, and to predict the theoretical outcome of a regeneration protocol if different explants were to be used.

Keywords Development · Explant · Growth correction factor · Geometric factor · Growth · Morphogenesis · Organogenesis

Electronic supplementary material The online version of this article (doi:10.1007/s00344-014-9437-x) contains supplementary material, which is available to authorized users.

J. A. Teixeira da Silva (✉)
P. O. Box 7, Miki-cho post office, Ikenobe 3011-2,
Kagawa-ken 761-0799, Japan
e-mail: jaimetex@yahoo.com

J. Dobránszki
Research Institute of Nyíregyháza, University of Debrecen,
P. O. Box 12, Nyíregyháza 4400, Hungary
e-mail: dobranszki@freemail.hu

The Thin Cell Layer: Concept and Application

The thin cell layer (or TCL) is an explant and, as the name suggests, a thin layer of cells usually measuring a few mm in thickness, but with variable proportions of length and diameter (Teixeira da Silva 2013). Undoubtedly, the explant is the most important biotic factor in plant tissue culture and its size, origin and age all determine its totipotency (the ability to regenerate a whole plant from any plant cell) and/or multipotency (the ability to derive organogenesis and regenerate any organ from any plant cell) in vitro under controlled environmental conditions imposed by a set of artificially imposed abiotic factors. Broadly, both concepts could be clustered into a single concept, the regeneration capacity (RC) of the explant.

The application of TCLs in basic and applied plant science has been covered in several comprehensive reviews

elsewhere (Teixeira da Silva 2003a, 2010, 2013; Nhut and others 2005, 2006; Teixeira da Silva and others 2007b; Teixeira da Silva and Tanaka 2010; Malabadi and Teixeira da Silva 2011; Teixeira da Silva and Dobránszki 2013a; and references therein). Apart from a mild challenge to the traditional nomenclature of the term by Teixeira da Silva in 2008, in which an alternate term, the thin tissue layer, or TTL, was suggested, the fundamental concept of the TCL has not changed at all, since it was first coined by Tran Thanh Van around 40 years ago using tobacco (*Nicotiana tabacum*) as the model plant (Tran Thanh Van 1973). Although this concept was almost never refined over a 40-year span, TCLs have seen a steady increase in use in over 140 papers in the past decade according to several data-bases.

The purpose of this study is not to review the literature, but to re-enforce some basic concepts of the TCL methodology that would allow plant scientists to apply it more widely in tissue culture experiments. Such a description does not exist, despite the use of TCLs since the early 1970s. The TCL, although a little bit more tedious to prepare and develop than a conventional explant due to its miniscule size and the fine-scale nature of the operation, can hypothetically yield several fold more organs than through the use of a conventional explant. This study will prove and quantify this concept in detail. TCLs have been used in basic and applied plant biotechnology, such as in characterizing genes involved in in vitro shoot organogenesis (Kim and Ernst 1994), in genetic transformation (for example, Teixeira da Silva 2005a, b), or in in vitro selection of rapeseed for Zn tolerance and accumulation (Ghnaya and others 2007). However, it is not known why a TCL is purportedly superior to a traditional explant despite several such claims in the literature, and never has this claim been theoretically or mathematically proved. Providing this proof is the focus of this study.

Preparing a tTCL and an lTCL

A TCL can be prepared from any explant source, provided that a blade sharp enough to cut a thin section less than 5 mm thick can be used. To date, most TCLs have been prepared by hand, and no study has ever documented the production of TCLs using a microtome. As shown in Fig. 1, a transverse TCL or tTCL and a longitudinal TCL or lTCL can be prepared from any type of tissue or organ. In the literature, tTCLs are sometimes referred to as thin cross sections or TCSs, whereas lTCLs are sometimes referred to as epidermal strips, thin epidermal layers or thin epidermal strips (reviewed in Teixeira da Silva 2013; Teixeira da Silva and Dobránszki 2013a). The important factor differentiating a TCL from a conventional explant is its surface to volume ratio, size and thickness (Fig. 1).

Organogenic Potential of Different Explant Types

To be able to measure or compare the RC (that is, morphogenic or organogenic potential) of an explant, two approaches are possible. The first (case I) is when the RC of one type of explant is compared to that of another, for example, a conventional explant versus a tTCL or an lTCL. For example, the number of shoots per regenerating explant. The second approach (case II) is when the yield of the source organ (or tissue) is considered using different explant types, that is, on a per-organ basis. For example, the number of shoots that regenerate from an organ, for example, from a leaf, petal, protocorm-like body (PLB), and so on. Five hypothetical examples in Annex 1 present different approaches to compare the RC of different explant types.

Why are two approaches (cases) considered? This is related to a practical situation, a plant researcher faces when conducting a tissue culture experiment. The first question that should arise is how to cut an explant, and with what shape and size to obtain the most regenerants? The other fundamental question is: how can the number of regenerants be affected (that is, increased) by manipulating the size of an explant with a given shape under the same experimental conditions? When a scientist observes regeneration, it is frequent that both the percentage of explants that regenerated organ(s) and the number of organ(s) per regenerating explant are measured. That means that there are two components for 'successful' regeneration, and therefore, there exist two levels of comparison, namely a per-regenerating-explant level (case I) and a per-source-organ level (case II). When the number of regenerated organ(s) is observed taking into consideration only explants that regenerated organ(s), the RC [that is, the ability of an explant to form organ(s)] is compared. This is the "per explant" comparison, that is, case I, or the yield of an explant. In other words, one conventional organ-regenerating explant is compared with one organ-regenerating tTCL (or other type of explant). If the objective is—and practically and finally it usually is—to define the most efficient explant size for a regeneration protocol, the percentage of explants that regenerate organs (*R* %) for shoots (SR %) or for PLBs (PLB %) must understandably also be taken into consideration. This is the "per organ" base (case II) comparison, that is, the yield of a source organ in which the number of organs that can be regenerated from the source organ is calculated (Annex 1).

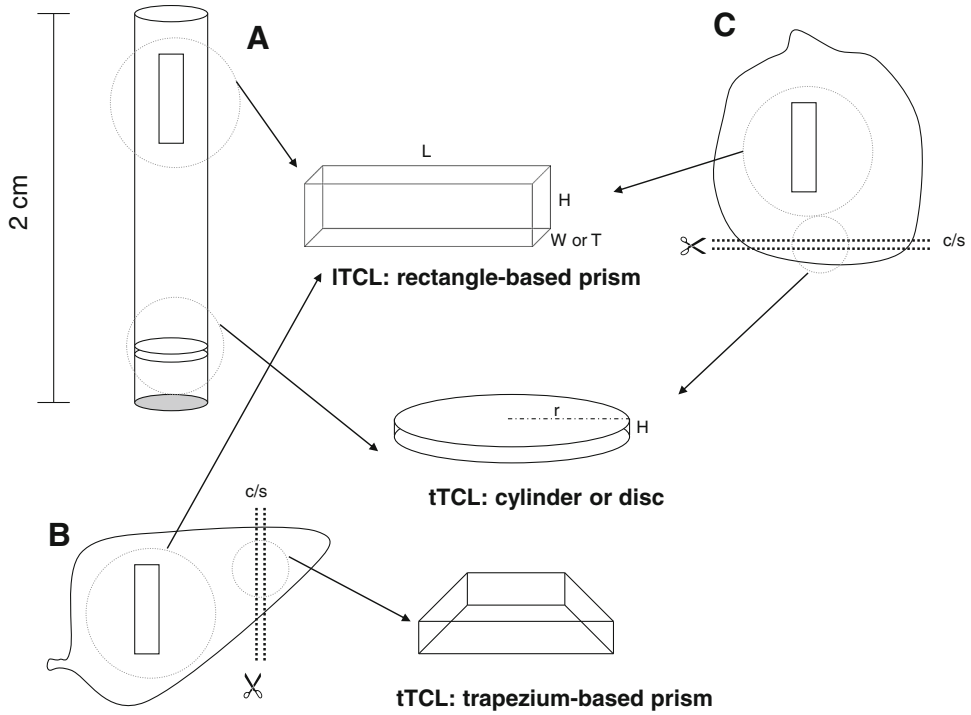

Fig. 1 Schematic diagram of how a transverse thin cell layer (tTCL) or a longitudinal thin cell layer (lTCL) can be produced from almost any explant source. **a** Typical sources include stem internode tissue, pedicels, peduncles, roots, and apical meristematic areas from any monocotyledonous or dicotyledonous plant. **b** Typical sources include leaves, petals and sepals from any monocotyledonous or dicotyledonous plant. **c** Typical sources include protocorm-like bodies as found in orchids, corms, cormlets, tubers or bulbs, or round or dome-like organs such as ovaries. For **a**, **b** and **c**, the lTCL explant is prepared from the surface (epidermal and subepidermal layers only) of any organ, whereas in the tTCL, the explant is prepared from a cross-section of any organ, thus cutting through several different cell/tissue layer types. *H* height, *L* length, *W* or *T* width or thickness, *r* radius, *c/s* cross-section through the tissue, *scissors* cut line during explant preparation leading to c/s. *2× c/s* 1× lTCL. *Cuboid* with rectangle-based prism; *trapezoid* trapezium-based prism. An explanation of the equations, sizes and proportions can be obtained from Table 3 and Annex 3

Introducing the Growth Correction Factor and Geometric Factor

The growth correction factor (GCF) is a proportional number that expresses how many times more target organs can be regenerated from a source organ in a comparison of two explants. GCF, therefore, is a novel concept that would allow for the true comparison of the RC of any explants derived from any plant source organs or tissues, such as a leaf, PLB, stem, root, apical meristem, and so at the (source) organ (or tissue) level (that is, case II). Provided that the same cultivar and experimental procedures are followed, the GCF would theoretically allow a scientist to compare the true RC of a genus, species or cultivar with what was already published in the literature in other protocols provided that an accurate account of the explant size was made available. Usually, this involves a detailed description of the protocol, including explant size and preparation. The initial hypothetical and philosophical basis of the GCF has been covered elsewhere (Teixeira da Silva and Dobránszki 2011). In theory, each cultivar or variety would have its own GCF for each explant type because explant type, position, age, size, as well as many other factors, such as basal medium, plant growth regulators (PGRs), sucrose, light, temperature, affect the RC. However, if we compare the RC of two explants, their size and shape are very important parameters. During a 'per-regenerating-explant' comparison (case I) there is a component of the RC of the explant that depends only on the size and shape of the explant and can be calculated based on its surface and volume. This is the geometric factor (GF). In Table 1, we describe how different explant types from apple (*Malus domestica* Borkh.), *Cymbidium* and chrysanthemum (*Dendranthema grandiflora* Kitamura) can be estimated by geometric shapes to determine their surface area or volume (Annexes 2, 3 and Figs. 2, 3, 4), because these explant parameters are important when comparing explants, depending on whether differentiation takes place from epidermal or subepidermal layers (Teixeira da Silva 2005b; Teixeira da Silva and Tanaka 2006) as in the case of *Cymbidium* and chrysanthemum or also from the mesophyll as occurs in apple (Dufour 1990; Pawlicki and Welander 1994).

Table 1 Geometric factor (GF) from actual data to potential regeneration potential of three species

Model crop	Explant source	Actual area (mm^2) of conventional explant[a]	Actual area (mm^2) + No. lTCLs per conventional explant	Actual area (mm^2) + No. tTCLs per conventional explant	Actual volume (mm^3) of conventional explant[a]	Actual volume (mm^3) of lTCLs	Actual volume (mm^3) of tTCLs	GF (lTCL compared to conventional/ tTCL compared to conventional)
Apple[b] 'Royal Gala'	Leaf	102.37[e]	No data available	7.81[e] + 25	9.59[e]	No data available	0.38[e]	No data/0.5192
Apple[b] 'Freedom'	Leaf	86.0[e]	No data available	5.03[e] + 25	7.06[e]	No data available	0.22[e]	No data/0.5328
Cymbidium[c]	PLB	37.68[f]	4.0 + 7	31.4[g] + 5	16.75	0.5	6.28	1.3333/0.6667
Chrysanthemum[d]	Stem internode	18.84	34	12.56	7.85	10	3.14	0.5/0.4

Shoot or PLB numbers were measured, surface areas and volumes were calculated from the measured size parameters of the explants and GF is calculated from the surface areas and volumes of the explants. Annexes 2 and 3 are applicable when the lTCL is made from a round organ to calculate the surface area

PLB protocorm-like body

[a] Conventional explants = 1/2 leaf (apple), 1/2 PLB (*Cymbidium*; Fig. 2), 1/2 stem internode (chrysanthemum; Fig. 3)

[b] *Malus domestica*

[c] *Cymbidium* hybrid Twilight Moon 'Daylight'

[d] *Dendranthema grandiflora* Kitamura cv. 'Shuhou-no-Chikara'

[e] Can be calculated from the surface area and volume of trapezium- or rectangle-based prism according to Fig. 4 and Table 3

[f] Calculated from the surface area and volume of a dome (that is, half a sphere) using r as 1.5 mm and π as 3.1416 = (1/2) 4 π r^2 = 2 π r^2 (http://www.monolithic.com/stories/dome-calculator; Annex 2 where the radius of a PLB is 1 mm (that is, diameter = 2 mm) and the height is 2 mm (Annex 2 and Table 3)

[g] Can be calculated according to Annex 3 and Table 3

Geometric factor is independent from any other in vitro experimental conditions, except for explant size and shape, and is one of the components in the comparison of RC on a 'per-regenerating-explant base'. GCF is the proportional number of target organs that can be regenerated from a source organ when comparing two explants that differ in size and/or shape, that is, comparison of RCs on a 'per-organ-base'. Consequently, GF should thus be proportional to GCF. The link between GF and GCF is the quotient of regeneration percentages (SR %s, PLB %, and so on, in general R %) of different explant types, but also takes into consideration the difference between the number of explants that can be prepared from a given organ:

$$R\,\%_{\mathrm{conv}}/n\,R\,\%_{\mathrm{tTCL}},$$

where n = the number of TCLs that can be theoretically prepared from a source explant.

The proportional factor k between GF and GCF can be different depending on other in vitro experimental conditions that affect the success of the regeneration process, such as medium, lighting, genotype, explant age, sampling time, and so on, and these factors are mathematically summarized as a k factor.

Therefore,

$$\mathrm{GCF} = \frac{n\,R\,\%_{\mathrm{tTCL}}}{R\,\%_{\mathrm{conv}}} k\mathrm{GF} \qquad (1)$$

If only one factor is different, for example, the cultivar, k is simply calculated. If, however, more factors change in an experiment, the new k results from these factors which affect the outcome of regeneration, that is, RC. In other words, k can only be determined experimentally in response to an experimental factor or a change in factor such as PGRs, light intensity or temperature. Therefore, when two explants are compared under the same experimental conditions (basal medium, PGRs, sucrose, light, temperature, and so on), but only the explant size or shape differ, then k is the same.

The following three sections describe how the GF and GCF can be calculated in three model species. Moreover, we explain how this calculation can be practically used to predict the RC in vitro if both conventional and TCL explants have a different shape and size.

Cymbidium

Cymbidium is a less well known, but well-established and excellent model species because organogenesis in vitro has also been extremely well established, primarily conducted in studies by the first author through the use of PLBs, callus and/or somatic embryogenesis (Teixeira da Silva and Tanaka 2006; Teixeira da Silva and others 2007a; Teixeira da

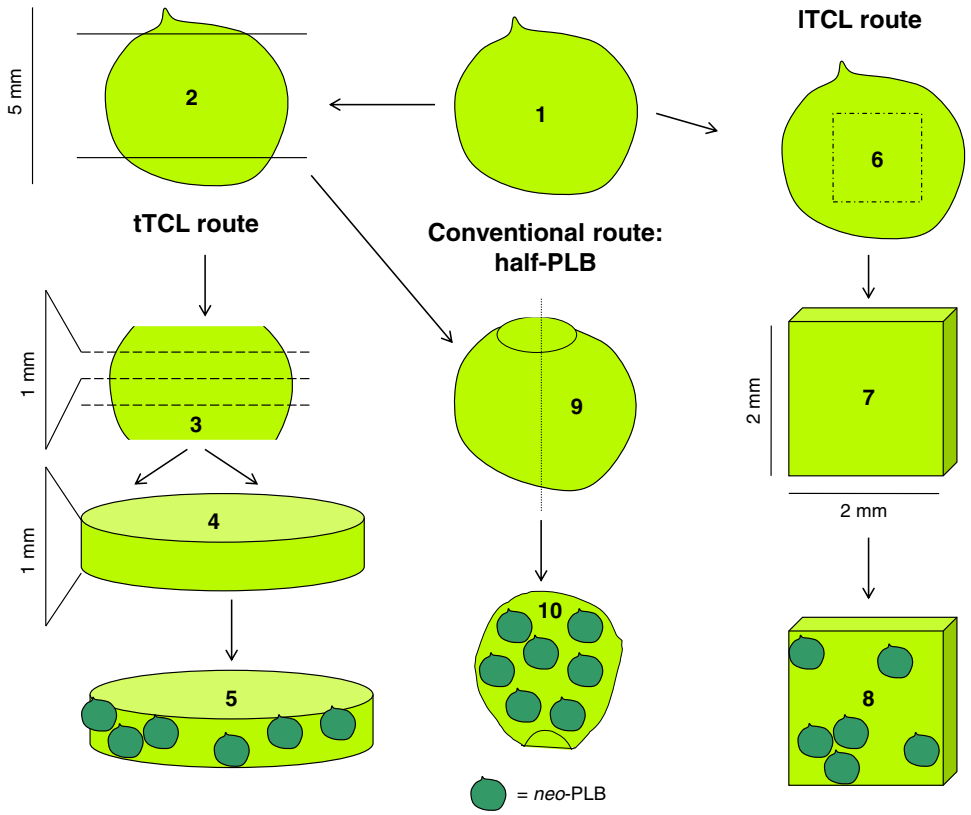

Fig. 2 Schematic diagram of how a conventional explant (half-PLB), a transverse thin cell layer (tTCL) or a longitudinal thin cell layer (lTCL) is produced for any *Cymbidium* cultivar, independent of the size of the protocorm-like body (PLB). (*1*) The donor explant is in fact a PLB that is 45–60 days old. (*2*) The actively dividing cells of the apical meristem and surrounding tissues, as well as the whitish-yellow poorly organogenic tissues at the base of the PLB (usually the part of the PLB in contact with solid, agarized medium) are sliced off with a feather-leaf blade. In the tTCL route, (*3*) only the central 2–3 slices of the PLB, covering the equator of the PLB, as well as its tropics, each 1 mm thick (*4*), are considered to be tTCLs. (*5*) Neo-PLBs, or new PLBs, form exclusively on the surface or epidermal tissue, and never from tissue within the inner part of the tTCL. In the lTCL route, on the other hand, (*6*) a square explant is cut along the surface of the PLB, usually 2 mm × 2 mm in size, yielding (*7*) 2-3 explants per original PLB. (*8*) Neo-PLBs form exclusively on the surface or epidermal tissue, and never from tissue within the inner (or under) part of the lTCL. In the conventional route, PLBs from which the apical meristem and basal tissues have been trimmed are bisected into two equally sized half-PLBs (*dome-shaped*) (*9*) which form neo-PLBs on the surface (*10*)

Silva 2012, and references therein). Using new Teixeira *Cymbidium* (TC) medium, specifically for cv. Twilight Moon 'Daylight', 8.3 PLBs could form per half-PLB explant (Teixeira da Silva 2012), but 6.4 PLBs could form from PLB tTCLs, and 3.6 PLBs could form from lTCLs (unpublished data) (Table 2). The number of PLBs forming from lTCLs and tTCLs of different genotypes also differs (Teixeira da Silva 2013).

In independent experiments, nine *Cymbidium* cultivars were studied. In *Cymbidium*, organogenesis occurs from epidermal or subepidermal cells, but not from the mesophyll cells (Teixeira da Silva and Tanaka 2006). Therefore, we hypothesize that not the whole surface area (A_{conv}, A_{tTCL}, A_{lTCL}) but only the epidermal surface area of an explant ($A_{conv,epid}$, $A_{tTCL,epid}$, $A_{lTCL,epid}$) may affect its RC beside its volume (V_{conv}, V_{tTCL}, V_{lTCL}). tTCL and lTCL explants were prepared from conventional PLBs, as shown in Fig. 2. The conventional explant (a half-PLB) is a dome or hemisphere, the tTCL is a cylinder or disc, whereas the lTCL is a rectangle-based prism, representing practically the epidermal and subepidermal layer of the PLB.

Geometric factor, which was determined both for tTCL (GF_{tTCL}) and lTCL (GF_{lTCL}) explants by comparing with the conventional explant (half-PLB), is proportional to the quotient of the PLBs on different explant types (Tables 1, 2), as follows:

$$PLB_{tTCL} = GF\,k\,PLB_{conv}, \quad (2a)$$

where PLB_{tTCL} and PLB_{conv} correspond to the number of PLBs per explant that develop on a tTCL (PLB_{tTCL}) and on a conventional explant (PLB_{conv}), respectively. k is a correction factor which may depend on several other biotic or abiotic factors during regeneration such as genotype,

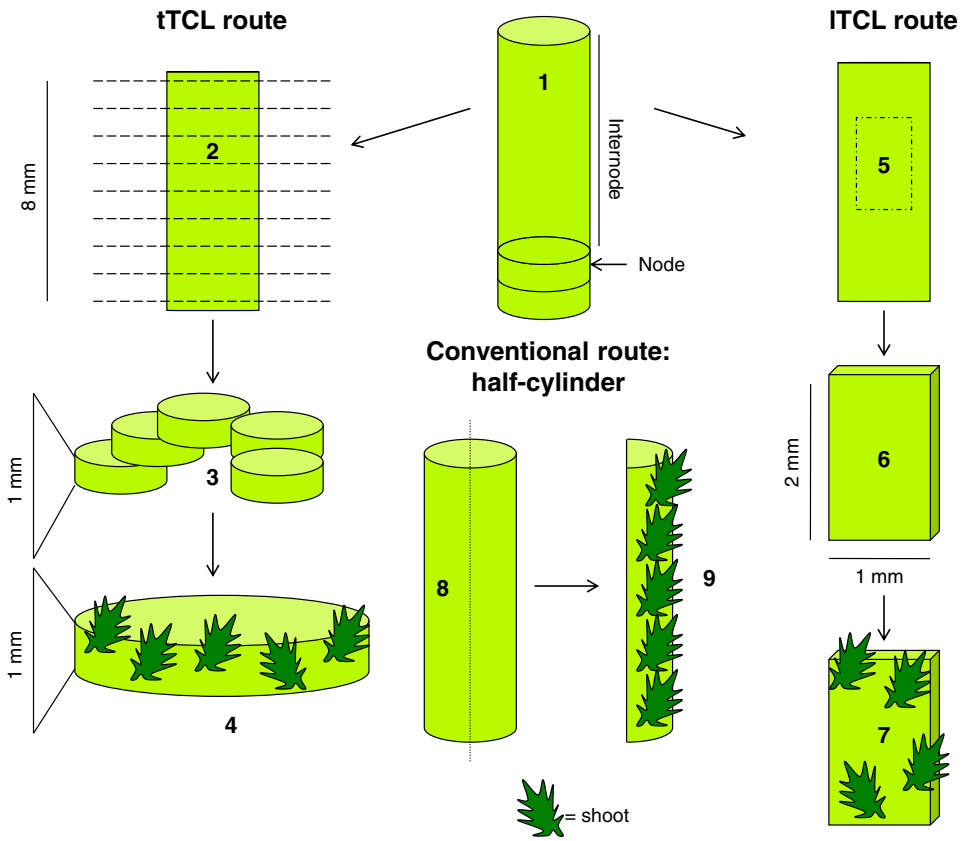

Fig. 3 Schematic diagram of how a conventional explant (stem internode), a transverse thin cell layer (tTCL) or a longitudinal thin cell layer (lTCL) is produced for any chrysanthemum cultivar. (*1*) The donor explant is an internode. In the tTCL route, (*2*) the internode can be relatively easily cut into rings 1-mm thick, in this case 8 tTCLs from a 10-mm long internode. (*3*) tTCLs should be maintained with the basal side down on medium as in the *in planta* condition. (*4*) Shoots form exclusively on or from the surface or (sub)epidermal tissue, and never from tissue within the inner part of the internode. In the lTCL route, on the other hand, (*5*) a rectangular explant is cut along the surface of the internode, usually (*6*) 2 mm (length) × 1 mm (width) in size, yielding 2 explants per 2–3 mm of internode tissue (total number depends on total length of internode. (*7*) Shoots form exclusively on or from the surface or (sub)epidermal tissue, and never from tissue within the inner part of the internode. In the conventional route, stem internodes are bisected into two equally sized halves (*half-cylinder*) (*8*) which form new shoots on the surface (*9*; orientation is cut surface down on medium)

variety, explant position, and so on, which are independent of the explant size and shape.

Cylinder-Shaped tTCL Compared to a Conventional Dome-Shaped Explant

$$\mathrm{GF_{tTCL}} = \frac{\frac{A_{\mathrm{tTCL,epid}}}{V_{\mathrm{tTCL}}}}{\frac{A_{\mathrm{conv,epid}}}{V_{\mathrm{conv}}}} \tag{3}$$

Using Table 3 and Fig. 2 and after substitution and simplifications (Annex 4), Eq. 3 can be expressed as:

$$\mathrm{GF_{tTCL}} = \frac{\frac{A_{\mathrm{tTCL,epid}}}{V_{\mathrm{tTCL}}}}{\frac{A_{\mathrm{conv,epid}}}{V_{\mathrm{conv}}}} = \frac{\frac{2\pi r_{\mathrm{tTCL}} h}{r_{\mathrm{tTCL}}^2 \pi h}}{\frac{2\pi r_{\mathrm{conv}}^2}{\frac{2\pi r_{\mathrm{conv}}^3}{3}}} = \ldots = \frac{2}{3}\frac{r_{\mathrm{conv}}}{r_{\mathrm{tTCL}}} \tag{4}$$

According to Eq. 4 it can be seen that GF depends only on the quotient of the explant's radius. Equation 4 is always true, independent of the germplasm, organ source and applied protocol, under two conditions: (1) if the conventional explant is dome-shaped; (2) if the tTCL explant is a cylinder or disc, and maintaining our initial assumption that regeneration occurs only from the epidermis.

In the present experiment, only the central part of the PLB is used to create a tTCL (Fig. 2), that is, in this case $r_{\mathrm{conv}} = r_{\mathrm{tTCL}}$, so:

$$\mathrm{GF_{tTCL}} = \frac{2}{3}$$

Based on Eqs. 1 and 2a, the quotient of $\mathrm{PLB_{tTCL}}$ and $\mathrm{PLB_{conv}}$:

$\frac{\mathrm{PLB_{tTCL}}}{\mathrm{PLB_{conv}}} = \mathrm{GCF}\frac{\mathrm{PLB\,\%_{conv}}}{n\,\mathrm{PLB\,\%_{tTCL}}} = k\,\mathrm{GF}$, in our experiments $n = 1$, and $\mathrm{GF_{tTCL}} = \frac{2}{3}$ so the equation becomes $\frac{\mathrm{PLB_{tTCL}}}{\mathrm{PLB_{conv}}} = \mathrm{GCF}\frac{\mathrm{PLB\,\%_{conv}}}{\mathrm{PLB\,\%_{tTCL}}} = k\frac{2}{3}$.

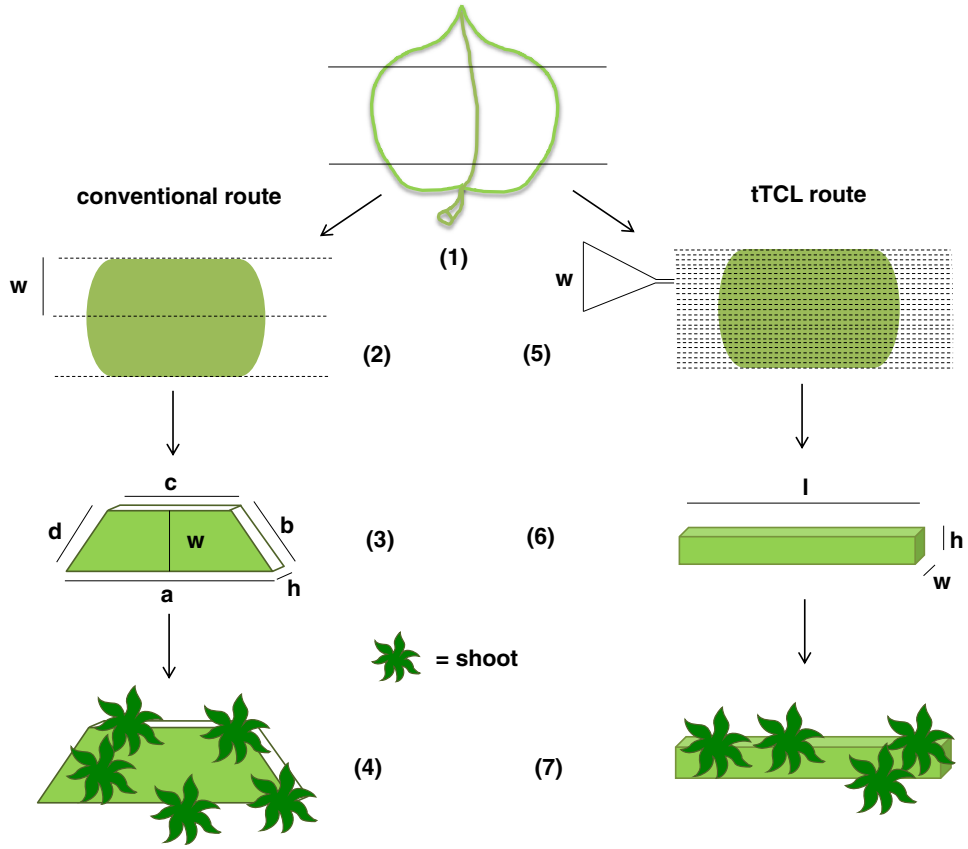

Fig. 4 Schematic diagram of how a conventional explant (leaf segment), and a transverse thin cell layer (tTCL) are produced for any apple cultivar. (*1*) The donor explant is a leaf. After removing the petiole and apex, (*2*) the leaf can be cut transversely into 2 strips [width (*w*) 5 mm] in the conventional route. (*3*) Conventional explants are maintained with the adaxial side down on medium. *Trapesium-based prism* with the indicated sizes (*a*, *b*, *c*, *d*, *w*, *h*) can be used for the geometric estimation of conventional leaf explants, where the *upper* and *lower bases* are *trapezoids* (uses explained in Table 3). (*4*) Shoots form both from the epidermal, subepidermal and from mesophyll cells in apple. In the tTCL route (*5*), the leaf can be cut transversely into 50 tTCL segments (*w* 0.1–0.3 mm) and (*6*) maintained with the adaxial side down on medium. For the geometric estimation of tTCL leaf explants rectangle-based prism with the indicated sizes (*l*, *w*, *h*) can be used, where *upper* and *lower bases* are *rectangles*. Even though tTCLs from a leaf could be *trapezium-based prisms* de facto, in reality, it is extremely difficult to measure the sizes, especially when we are dealing with explants 0.5–2 mm in size. Therefore, tTCLs from any non-round or non-cylindrical organ have been classified as rectangle-based prisms, where '*w*' is the thickness of the explants (uses explained in Table 3). (*7*) Shoots form both from the epidermal, subepidermal and from mesophyll cells in apple

In these experiments with *Cymbidium*, only the cultivars were different, therefore, the value of k in Eq. 2a depends only on cultivar. The values of k varied between 0.2 and 1.2 in the case of tTCL explants versus conventional explants (Tables 4, 5).

Rectangle-Based Prism-Shaped lTCL Compared to a Conventional Dome-Shaped Explant

Using Table 3 and Fig. 2, and after substitution and simplifications (Annex 4), Eq. 3 can be expressed as:

$$\mathrm{GF_{lTCL}} = \frac{\frac{A_{\mathrm{lTCL,epid}}}{V_{\mathrm{lTCL}}}}{\frac{A_{\mathrm{conv,epid}}}{V_{\mathrm{conv}}}} = \frac{\frac{lw}{lwh_{\mathrm{lTCL}}}}{\frac{2\pi r^2_{\mathrm{conv}}}{\frac{2\pi r^3_{\mathrm{conv}}}{3}}} = \ldots = \frac{r_{\mathrm{conv}}}{3h_{\mathrm{lTCL}}} \quad (5)$$

According to Eq. 5, it can be seen that the GF depends on the thickness of the lTCL and on the radius of a conventional explant (Table 3).

Equation 5 is always true under this condition: if the lTCL is prepared from and compared to a conventional explant with a dome shape, and maintaining the initial assumption that regeneration occurs only from the epidermis.

As in the tTCL-conventional explant comparison ("Cylinder-shaped tTCL compared to a conventional half-cylinder-shaped explant" section), based on Eqs. 1 and 2a, the quotient of $\mathrm{PLB_{tTCL}}$ and $\mathrm{PLB_{conv}}$ gives $\frac{\mathrm{PLB_{tTCL}}}{\mathrm{PLB_{conv}}} = \mathrm{GCF}\frac{\mathrm{PLB\ \%_{conv}}}{n\,\mathrm{PLB\ \%_{tTCL}}} = k\,\mathrm{GF}$, in our experiments, PLB % in both

explant types is 100 %. Therefore, using Eqs. 5 and 1: $GCF = nk\frac{r_{conv}}{3h_{lTCL}}$ from $\frac{PLB_{lTCL}}{PLB_{conv}} = \frac{GCF}{n} = k\frac{r_{conv}}{3h_{lTCL}}$.

In these experiments with *Cymbidium* only the cultivars were different, therefore the value of k from the above equation depended only on cultivar as mentioned earlier. The values of k ranged between 0.06 and 0.32 in the case of lTCL explants versus conventional explants (except when zero PLBs regenerate, then $k = 0$) (Tables 4, 5).

Table 2 Comparison between the shoot regeneration potential of three model plant species using thin cell layers (TCLs) versus conventional explants from which the TCLs are derived

Model crop	Explant source	No. of shoots/explant		
		Conventional explant[d]	lTCL	tTCL
Apple[a] 'Royal Gala'	Leaf	12.1	No data available	6.5
Apple[a] 'Freedom'	Leaf	3.2	No data available	2.4
Cymbidium[b]	Half-PLB	8.3	3.6	6.4
Chrysanthemum[c]	Stem internode	4.5	2.3	1.2

PLB protocorm-like body

[a] *Malus domestica* cultivars 'Royal Gala' and 'Freedom'

[b] *Cymbidium* hybrid Twilight Moon 'Daylight'

[c] *Dendranthema grandiflora* Kitamura cv. 'Shuhou-no-Chikara'

[d] Conventional explants = 1/2 leaf (apple), 1/2 PLB (*Cymbidium*), 1/2 stem internode

Chrysanthemum

Chrysanthemum is also a suitable model species for in vitro studies due to its ability to respond easily in vitro. Regeneration protocols for chrysanthemum have also been extremely well developed and characterized, including through the use of TCLs (Teixeira da Silva 2003b; Teixeira da Silva 2004; Teixeira da Silva 2005b; Teixeira da Silva 2005a, b, 2012; and references therein). In chrysanthemum cv. 'Shuhou-no-Chikara', using a newly defined Teixeira's chrysanthemum shoot growth medium (TCSGM) (Teixeira da Silva 2014), stem internode explants, tTCLs and lTCLs can form 4.5, 2.3 and 1.2 shoots/explant, respectively (Table 2). In both plants, at face value, what is evident is that conventional explants 'appear' to form more PLBs or shoots for *Cymbidium* and chrysanthemum, respectively, than tTCLs and lTCLs.

Due to its market popularity, one chrysanthemum cultivar was studied, namely 'Shuhou-no-Chikara'. In chrysanthemum, organogenesis occurs from epidermal or subepidermal cells, but not from mesophyll cells (Teixeira da Silva 2005b). Therefore, as in *Cymbidium*, we hypothesized that the epidermal surface area of an explant ($A_{conv,epid}$, $A_{tTCL,epid}$, $A_{lTCL,epid}$) might play a role in the RC of the explants. tTCL and lTCL explants were prepared from conventional internodes, as presented in Fig. 3. The conventional explant was a half-cylinder, the tTCL was a cylinder or disc, whereas the lTCL was a rectangle-based prism. In the latter case, the entire explant was assumed to be the epidermal and subepidermal layer of the internode, since no regeneration was observed from inner tissues.

Table 3 Surface area and volume of differently shaped TCLs and conventional explants estimated by geometric shapes

Geometric solid	Surface (A)	Surface covered by epidermis (A_{epid})	Volume (V)	Explant type
Rectangle-based prism	$A = 2\ h\ (l + w) + 2\ lw$	$2\ lw$ (in tTCLs) lw (in lTCLs)	$V = lwh$	tTCL from leaf[b]; lTCLs from any organ
Cylinder/disc	$A = 2\ \pi r\ (h + r)$	$2\ \pi rh$	$V = r^2\pi h$	Conventional stem explants; tTCL from round or cylindrical organs, such as stem, root, pedicel, peduncle
Half-cylinder	$A = \pi r\ (h + r)$	πrh	$V = (r^2\pi h)/2$	Conventional stem explants; tTCL from round or cylindrical organs, such as stem, root, pedicel, peduncle
Trapezium-based prism[a]	$A = h\ (a + b + c + d) + w\ (a + c)$	$2\ w\ (a + c)$	$V = h\ (w\ (a + c)/2)$	Conventional leaf explant
Dome (hemisphere)	$A = 3\ \pi r^2$	$2\ \pi r^2$	$V = 2/3\ \pi r^3$	Whole or half-PLB, SAM

[a] a, b, c, d: Length of the sides of a conventional leaf segment resembles a trapezium, where a and c are the opposite sides, that is, cut sides of the trapezium (for example, leaf segments) (see Fig. 4 for explanations and abbreviations)

[b] Practically speaking, even though tTCLs from a leaf could be trapezium-based prisms de facto, in reality, it is extremely difficult to measure the sizes, especially when we are dealing with explants 0.5–2 mm in size. Therefore, tTCLs from any non-round or non-cylindrical organ have been classified as rectangle-based prisms, where 'w' is the thickness of the explants

PLB protocorm-like body, *SAM* shoot apical meristem (shoot tip)

Table 4 Inter-cultivar variation in *Cymbidium* hybrid *neo*-PLB formation depending on explant size and surface area. Number of PLBs formed can be compared after applying the GCF

Cymbidium cultivar[a]	No. PLBs/explant[b]			Ratio of No. PLBs/explant (Half-PLB:tTCL:lTCL)
	Half-PLB[c,d]	tTCL[c]	lTCL[c]	
246-2	8.3	6.4	3.6	0.45:0.35:0.2
653-2	7.2	2.8	1.1	0.65:0.25:0.10
649-4	6.4	3.4	1.3	0.58:0.31:0.12
485-12	4.6	2.3	1.2	0.57:0.28:0.15
167-1	3.2	1.1	0.4	0.68:0.23:0.09
553-1	3.1	2.6	0.8	0.48:0.40:0.12
91-8	2.6	0.4	0	0.87:0.13:0
536-1	2.2	0.8	0.3	0.67:0.24:0.09
204-1	1.8	0.2	0	0.90:0.10:0

[a] Cultivar crosses (BioU classification): 91-8 (Aroma Candle 'Hot Heart') = Jenteel 'Pair Look' X Seaside 'Crown Princess'; 167-1 (Pretty Poetry 'Malachite') = Mini Sarah 'Artisan' X Eastern Star 'Green Fields'; 204-1 (Alice Beauty 'No. 1') = Alice Luna X Sleeping Beauty 'Mistuko'; 246-2 (Twilight Moon 'Day Light') = Lovely Bunny 'Romeo' X Hiroshima Golden Cup 'Sunny Moon'; 485-12 (Spring Night 'No. 12') = Tiny Sour X Twilight Moon 'Day Light'; 536-1 (Dream City 'No. 1') = Great Katy 'Tender' X Lucky Flower 'Anmitsuhime'; 553-1 (Call Me Love 'Snow Princess') = Jenteel 'Pair Look' X Great Katy 'Tender'; 649-4 (Energy Star 'No. 4') = Morning Moon 'Great Tiger' X Twilight Moon 'Day Light'; 653-2 (Sweet Moon 'No. 2') = Yellow Candy 'Lemon Fresh' X Twilight Moon 'Day Light'. All cultivars courtesy of BioU, Tokushima, Japan

[b] $n = 30$ (10 × 3)

[c] Prepared according to Teixeira da Silva and Tanaka (2006) on Teixeira *Cymbidium* (TC) 1 medium (Teixeira da Silva 2012). Half-PLBs are the conventional explant for *Cymbidium*

[d] Cultivars are ranked based on proliferation level (highest to lowest) of half-PLBs

The average diameter of an intact PLB for each cultivar is as follows: 6 mm (653-2, 649-4, 485-12, 167-1), 4 mm (246-2, 553-1, 536-1), 2 mm (91-8, 204-1)

Geometric factor was determined both for tTCLs and lTCLs, and always represents a comparison with the conventional explant.

Cylinder-Shaped tTCL Compared to a Conventional Half-Cylinder-Shaped Explant

The quotients of the epidermal surface (A_{epid}) and the volume of conventional and tTCL explants are equal, because (Table 3; Annex 4):

$$\frac{A_{tTCL,epid}}{V_{tTCL}} = \frac{2\pi r h_{tTCL}}{r^2 \pi h_{tTCL}} = \frac{2}{r}$$

Similarly, $\frac{A_{conv,epid}}{V_{conv}} = \frac{\pi r h_{conv}}{\frac{r^2 \pi h_{conv}}{2}} = \frac{2}{r}$

Therefore, their quotients did not play a role in the different RCs of the two explant types.

In this case, GF was determined as the proportion of the epidermal surfaces of the two explant types, as follows:

$$GF_{tTCL} = \frac{A_{tTCL,epid}}{A_{conv,epid}} \quad (6)$$

Using Table 3 and knowing that $r_{conv} = r_{tTCL}$, therefore, $r_{conv} = r_{tTCL} = r$ and after substitution and simplifications (Annex 4):

$$GF_{tTCL} = \frac{A_{tTCL,epid}}{A_{conv,epid}} = \frac{2\pi r h_{tTCL}}{\pi r h_{conv}} = \ldots = \frac{2h_{tTCL}}{h_{conv}} \quad (7)$$

According to Eq. 7, it can be seen that GF depends on the lengths of both explant types (h_{conv}, h_{tTCL}) because the radius of the two explants is equal.

Equation 7 is always true, if the shape of the conventional explant is a half-cylinder and the shape of the tTCL, which is prepared from a half-cylinder with the same radius, is a disc, and maintaining our initial assumption that regeneration occurs only from epidermal or subepidermal cells and not from the mesophyll cells.

Considering Eq. 2a, the quotient of SN_{lTCL} and SN_{conv} is the same as the quotient of the PLBs on different explant types, such that

$$\frac{SN_{TCL}}{SN_{conv}} = k\,GF \quad (2b)$$

Based on Eqs. 1 and 2b, the quotient of SN_{lTCL} and SN_{conv} becomes:

$$\frac{SN_{TCL}}{SN_{conv}} = GCF\frac{SR\,\%_{conv}}{n\,SR\,\%_{TCL}} = k\,GF$$

Moreover, because SR % of both explant types is the same (100 %), $GCF = n\ k\ GF$. The value of n is 5, therefore, for chrysanthemum cultivar 'Shuhou-no-Chikara' $\frac{1.2}{4.5} = \frac{GCF}{5}$ (Table 2), which is the same as $GCF = \frac{6}{4.5} = 1.33$. GF is 0.4 from Table 1. From $GCF = n\ k\ GF$, k can be determined for this cultivar: $1.33 = 5\ k\ 0.4$ and $k = 0.667 \approx 0.7$.

Rectangle-Based Prism-Shaped lTCL Compared to a Conventional Half-Cylinder-Shaped Explant

$$GF_{lTCL} = \frac{\frac{A_{lTCL,epid}}{V_{lTCL}}}{\frac{A_{conv,epid}}{V_{conv}}} \quad (8)$$

Using Table 3 and after substitution and simplifications (Annex 4):

$$GF_{lTCL} = \frac{\frac{A_{lTCL,epid}}{V_{lTCL}}}{\frac{A_{conv,epid}}{V_{conv}}} = \frac{\frac{lw}{lwh_{lTCL}}}{\frac{\pi r h_{conv}}{\frac{r^2 \pi h_{conv}}{2}}} = \ldots = \frac{r}{2h_{lTCL}} \quad (9)$$

Table 5 Measured (number of PLBs) and calculated (GF, *k*, GCF) parameters from *Cymbidium neo*-PLB formation experiments (that is, case II)

Cultivar	Explant type	Number of PLBs regenerated[a]	GF (geometric factor)	*k* (correction factor)	GCF[b] (growth correction factor)
246-2	Conventional	8.3			
	tTCL	6.4	0.6667	1.2	0.8
	lTCL	3.6	1.3333	0.32	10.75
553-1	Conventional	3.1			
	tTCL	2.6	0.6667	1.2	0.8
	lTCL	0.8	1.3333	0.19	6.32
536-1	Conventional	2.2			
	tTCL	0.8	0.6667	0.5	0.33
	lTCL	0.3	1.3333	0.10	3.33
653-2	Conventional	7.2			
	tTCL	2.8	0.6667	0.6	0.4
	lTCL	1.1	2	0.08	4
649-4	Conventional	6.4			
	tTCL	3.4	0.6667	0.8	0.53
	lTCL	1.3	2	0.10	5
485-12	Conventional	4.6			
	tTCL	2.3	0.6667	0.8	0.53
	lTCL	1.2	2	0.13	6.5
167-1	Conventional	3.2			
	tTCL	1.1	0.6667	0.5	0.33
	lTCL	0.4	2	0.06	3
91-8	Conventional	2.6			
	tTCL	0.4	0.6667	0.2	0.13
	lTCL	0	0.6667	0	0
204-1	Conventional	1.8			
	tTCL	0.2	0.6667	0.2	0.13
	lTCL	0	0.6667	0	0

[a] Data from Table 4

[b] GCF, calculated according to Eqs. 1 and 2a, as the percentage of explants forming PLBs (PLB %) on different explant types, was the same within a cultivar [that is, the percentage of regenerating explants (compared to the number of explants prepared) was always 100 % or the same in the case of all explant types, that is conventional, tTCL, lTCL and all explants which were prepared regenerated]

According to Eq. 9, it can be seen that GF depends on the radius of the conventional explant and on the thickness of the lTCL (Table 3).

Equation 9 is always true if an lTCL is prepared from and compared to a conventional explant with the shape of a half-cylinder, and maintaining our initial assumption that regeneration occurs only from epidermal or subepidermal cells and not from mesophyll cells.

As for the *Cymbidium* tTCL, based on Eqs. 1 and 2b, the quotient of SN_{lTCL} and SN_{conv} becomes:

$$\frac{SN_{TCL}}{SN_{conv}} = GCF\frac{SR\,\%_{conv}}{n\,SR\,\%_{TCL}} = k\,GF.$$

Moreover, because (1) SR % of both explant types is the same (100 %) in this cultivar ('Shuhou-no-Chikara'), that is, GCF = *nk*GF, (2) the value of *n* was *n* = 1 therefore, GCF = *k*GF and (3) from the SN data (Table 2), the quotient of the SNs of two explant types was equal to GF, therefore, *k* was for this cultivar can be determined as *k* = 1.

Apple

Apple is a well-established and excellent model species because organogenesis in vitro is extremely well studied, defined and documented (Dobránszki and Teixeira da Silva 2010; Magyar-Tábori and others 2010), even though it is a hardwood species. The TCL is an excellent model for studying fine-scale organogenesis in apple and other species (Dobránszki and Teixeira da Silva 2011; Teixeira da Silva and others 2007b). In apple, shoot regeneration from in vitro leaves of 'Greensleeves' was dependent on the shape of the excised explant. James and others (1988), comparing the RC of 7-mm leaf discs, leaves cut lengthwise into three strips and leaves cut lengthwise into five strips and leaves cut transversely into three strips, noted higher regeneration when leaves were cut into strips than into discs and they hypothesized that this was because a greater surface area was cut per leaf using strips than when

discs were used, although these authors failed to quantify the size of explants. Early histological examinations (Welander 1988; Pawlicki and Welander 1994; Caboni and others 1996) had already proved that in shoot regeneration from in vitro leaves that not only epidermal or subepidermal cells but also mesophyll cells were active and that meristematic groups (supposedly equivalent to meristemoids) were detected after 3–6 days from all three cell layers. However, no information exists on whether there are any differences between cultivars regarding the degree of regeneration from mesophyll cells. Moreover, Dufour (1990) tested, in his co-cultivation experiments, whether endogenous hydrosoluble compounds could affect organogenesis by inducing regeneration in non-yielding genotypes or repress it in high-yielding ones. Dufour's results indicated that an as-yet-unidentified diffusible factor from a high-yielding genotype ('Gala') was able to improve the RC of a non-yielding genotype ('Golden Delicious').

Based on the global apple literature (Dobránszki and Teixeira da Silva 2010), we hypothesize that both the surface area and volume of an explant may affect its RC. Examining the data from our apple regeneration experiments with conventional or tTCL explants (Fig. 4) (Dobránszki and Teixeira da Silva 2013) using an easy-to-regenerate cultivar, 'Royal Gala' and a difficult to regenerate cultivar, 'Freedom' we concluded that practically the same surface areas were cut in both explant types using leaves as the source. In our highlighted examples from these experiments, this equates to, on average, 3.53 mm^2 in conventional explants and 3.99 mm^2 in tTCL explants from the second leaf of 'Royal Gala' and on average 2.91 mm^2 in conventional explants and 2.30 mm^2 in tTCL explants from the first leaf of 'Freedom'. This result suggests that the cut area of the explants did not play a role in the difference between the RC of different explants. However, considering that shoot regeneration from in vitro leaves was proven to occur both from epidermal or subepidermal and mesophyll cells (Welander 1988; Pawlicki and Welander 1994; Caboni and others 1996), the whole surface area (A_{conv} and A_{tTCL}), as well as the volume (V_{conv} and V_{tTCL}) of the explant were taken into consideration (Table 1). To be more exact, when the proportion of this quotient takes into consideration the two explant types, we can obtain a GF that is proportional to the quotient of the shoot number (SN) on different explant types (Tables 1, 2), according to Eq. 2b:

$$SN_{tTCL} = GF\, k\, SN_{conv},$$

where SN_{tTCL} and SN_{conv} correspond to the number of shoots per explant that develop on a tTCL (SN_{tTCL}) and on a conventional explant (SN_{conv}), respectively, similar to chrysanthemum.

In the case of a conventional apple leaf explant, a trapezium-based prism was used to estimate and calculate the surface area and volume of the explants, whereas in the leaf tTCL explant, a rectangle-based prism was used. Using Table 3, GF can be calculated as follows:

$$GF_{tTCL} = \frac{\frac{A_{conv}}{V_{conv}}}{\frac{A_{tTCL}}{V_{tTCL}}} = \frac{\frac{h_{conv}(a+b+c+d)+w_{conv}(a+c)}{h_{conv}\frac{w_{conv}(a+c)}{2}}}{\frac{2h_{tTCL}(l+w_{tTCL})+2lw_{tTCL}}{lw_{tTCL}h_{tTCL}}} \quad (10)$$

It is important to keep in mind, our initial assumption that regeneration occurs both from epidermal, subepidermal and mesophyll cells, and that GF is a fixed component of the comparison of regenerating explants in the sense that Eq. 10 is always true, if an explant with a shape of rectangle-based prism is compared to another explant with a trapezium-based prism shape. This is always true if regeneration takes place from the epidermis and mesophyll.

In Eq. 2b, $SN_{tTCL} = GF\ k\ SN_{conv}$, k is a correction factor which may depend on several circumstances or conditions during the regeneration experiments, which are independent of explant size and shape, but can affect the success of the regeneration process. These factors are mathematically summarized as a k factor. In our recently published experiments (Dobránszki and Teixeira da Silva 2011, 2013; Teixeira da Silva and Dobránszki 2013b), we studied four of these factors in apple: genotype, duration of the regeneration period (that is, sampling time), age/position of the explant and the effect of thidiazuron (TDZ) concentration in medium on the RC of tTCL explants (Tables 1, 2, 6; Annex 5). From these experiments regarding k, the following can be seen (Annex 5):

In response to the cytokinin-like compound, TDZ, conventional apple leaf explants could produce a maximum of 12.1 shoots per explant in 'Royal Gala' after 9 weeks of culture on medium containing 0.5 μM TDZ (Dobránszki and Teixeira da Silva 2013). In that experiment, the explant was a half-leaf strip 5-mm wide derived from the second leaf from the apex. However, when a 0.1–0.3 mm thick tTCL was used from the exact same leaf source, and from the same scion (cultivar), and placed on medium with the same concentration of TDZ, that is, 0.5 μM, only 4.1 shoots formed. Using Eq. 10, the value of k is 0.7. tTCL explants regenerated on medium with 5 μM of TDZ, which is an optimized concentration for tTCL explants derived from the second apical leaf of 'Royal Gala', 6.5 shoots could be produced per tTCL. In this case, using Eq. 10, the value of k is 1.0. Examining the leaf explants that originated from the first apical leaf of the same cultivar, we conclude that TDZ concentration did not significantly affect SN on tTCL explants. It was 5.5 using 0.5 μM TDZ, the same TDZ concentration that was applied for conventional explants ($SN_{conv} = 10.2$), and 5.1 using 5 μM TDZ in the medium for tTCL explants. Therefore, the value of k was also the same, that is, 1.1, in both comparisons (Annex 5).

Table 6 Measured (SN, SR %) and calculated (GF, *k*, GCF) parameters from apple regeneration experiments

Cultivar	Explant type	Position of the source leaf	SN (shoot number per regenerating explant)				GF (geometric factor)	*k* (correction factor)				SR % (percentage of explants forming shoots)				GCF (growth correction factor)			
Sampling time (week)			4	5	6	7	9		4	5	6	7	9	4	5	6	7	9	4	5	6	7	9
Royal Gala	Conventional	1st	1.0	1.3	2.4	6.3	10.2	0.4712	2.1	1.6	1.6	1.5	1.1	20	32	95	100	100	18.8	16.8	10.9	10.1	8.8
		2nd	1.0	1.2	3.0	7.6	12.1	0.5192	1.9	2.1	1.3	1.1	1.0	20	63.3	98	100	100	10.0	9.0	5.9	12.7	11.8
	tTCL	1st	1.0	1.0	1.8	4.4	5.1							15	28	55	58	70					
		2nd	1.0	1.3	2.1	4.4	6.5							8	21	33	88	88					
Freedom	Conventional	1st	0.0	1.0	1.5	2.2	3.2	0.5328	0.0	1.8	1.5	1.8	1.4	0	16.7	42	71	78	0.0	25.7	15.2	17.5	13.0
		2nd	0.0	1.0	1.6	2.3	2.6	0.5299	0.0	0.0	1.2	1.9	2.0	0	6.8	20	42	55	0.0	0.0	3.9	6.5	6.9
	tTCL	1st	0.0	1.0	1.2	2.1	2.4							0	18	32	52	54					
		2nd	0.0	0.0	1.0	2.3	2.8							0	0	5	11	14					

SN and SR % data were published in recent publications (Dobránszki and Teixeira da Silva 2013; Teixeira da Silva and Dobránszki 2013b) in other contexts

In Table 6, the values of k are calculated over sampling time if TDZ concentration of the medium was optimized both for conventional and tTCL explants (Teixeira da Silva and Dobránszki 2013b) for two scions and for two positions of the source leaf. Table 6 demonstrates how the value of k can change if cultivar, sampling time and the position of the source explant change in an experiment.

Further investigations examining the role of other potentially important factors that affect the regeneration ability of an explant may enable a more exact explanation and/or mathematical description of the k factor. In other words, the greater the number of influencing factors, the greater the number of components that affect k, with each influencing factor representing a separate subset of the k factor. That means practically for instance, that if we use the same experimental protocol, but with a different cultivars, we could be able to determine the role of the cultivar in the k factor, because from Eq. 2b, that is $\frac{SN_{tTCL}}{SN_{conv}} = k\,GF$, GF is a constant (independent of the cultivar), and $\frac{SN_{tTCL}}{SN_{conv}}$ can be determined from the experiments for both cultivars, but k will be different for each cultivar provided the examined cultivars have different RCs.

Growth correction factor was calculated in apple leaves as described earlier (Teixeira da Silva and Dobránszki 2011; Dobránszki and Teixeira da Silva 2013): $[25 \times (SR\ \%_{tTCL} \times SN_{tTCL})/100]/[(SR\ \%_{control} \times SN_{control})/100]$. In our example on apple (Dobránszki and Teixeira da Silva 2013b) (Table 2, 6), this means that in 'Royal Gala' when explants originated from the second apical leaf of in vitro shoots, for one leaf, 24.2 shoots formed using conventional explants (SN 12.1, SR % 100 %), whereas 286 shoots formed using tTCL explants (SN 6.5, SR % 88 %). Thus, the GCF is 11.8. In 'Freedom', when explants originated from the first apical leaf of in vitro shoots, 4.99 shoots per leaf regenerated using conventional explants (SN 3.2; SR % 78 %) and 64.8 shoots per leaf using tTCL explants (SN 2.4, SR % 54 %). So, the GCF is 13. The RC of 'Freedom' can be increased (GCF of 13 vs. GCF 11.8 in 'Royal Gala) more than that of 'Royal Gala' using adequate explants, in this case tTCL explants instead of conventional (half-leaf with 5-mm thickness) explants. From the above equation and using Eq. 1, the quotient of SN_{tTCL} and SN_{conv} becomes: $\frac{SN_{tTCL}}{SN_{conv}} = GCF\frac{SR\,\%_{conv}}{n\,SR\,\%_{tTCL}}$, that is in the case of apple leaves: $\frac{SN_{tTCL}}{SN_{conv}} = GCF\frac{SR\,\%_{conv}}{25\,SR\,\%_{tTCL}}$

Moreover, considering Eq. 2b: $\frac{SN_{tTCL}}{SN_{conv}} = GCF\frac{SR\%_{conv}}{nSR\%_{tTCL}} = k\,GF$, that is, $\frac{SN_{tTCL}}{SN_{conv}} = GCF\frac{SR\%_{conv}}{nSR\%_{tTCL}} = k\frac{\frac{A_{conv}}{V_{conv}}}{\frac{A_{tTCL}}{V_{tTCL}}}$.

How can the GF and GCF be Employed in Practical Terms?

This study has two key objectives. First, it aims to precisely quantify regeneration from three model species using explants whose shape and size (and thus area and volume) are clearly defined. Using actual regeneration data—shoots in the case of apple and chrysanthemum and PLBs in the case of *Cymbidium*—it is then possible, using the GF and GCF, to compare their organogenic potential where the two explants being compared have the same size and/or area/volume or even two explants that have two sizes. Why is this important? As was initially discussed in an earlier elaboration of our theory (Teixeira da Silva and Dobránszki 2011), one of the greatest weaknesses of the plant tissue culture literature is the lack of details in tissue culture protocols outlying the exact shape and size of explants. Without this basic information, it is difficult to directly compare protocols between studies, between genera, or between laboratories, which is the second key objective of this study. The reason is primarily, in many instances, because the precise explant size and shape are not explained in such papers. One of the unfortunate or unintended consequences is that studies in plant tissue culture may make unsupported claims using the wording 'our protocol shows that regeneration was higher than that reported by XYX and others' or 'explant A in our study produced more organs than explant B in XYX and others' study'. What our manuscript indicates is that, from now on, unless the exact size and area of an explant is not indicated by both studies, no direct comparison can truly be made, and thus no conclusion regarding superiority of the protocol can be reached, or assumed. In a way, the GCF introduces a new form of quality control into the plant tissue culture literature, forcing authors to report the exact explant size and shape so that a GCF can be calculated by any other scientist and not simply to automatically adopt a protocol by another manuscript to their own cultivar or germplasm without assessing first the optimal size and shape for maximum regeneration.

A plant tissue culture scientist might ask, in response to this paper, how can I express my RC and the GCF in my own experiment? This might best be explained by apple data in Annex 5. Under the same experimental conditions, by changing the explant size and hereby preparing 50 tTCL explants instead of 2 conventional explants from the source organ (1st or 2nd apical in vitro leaf), the GCF can be increased 2.9- or 2.6-fold (depending on the position of the source leaf) after a 9-week-long culture period. The efficacy of the protocol can be further increased by optimizing the PGR concentration, that is, TDZ content of the medium to the demand of that new explant type, that is, tTCL. There was a subsequent increase in the GCF (RC) from 2.9 to 8.8 and from 2.6 to 11.8 depending on the position of the source leaf.

Summary

This study provides the first quantitative means to assess the real regeneration potential of an explant, allowing it to be compared to another explant, from another study, another plant or another laboratory, provided that the explant size and shape are accurately reported in both studies.

Two concepts, the GF and the GCF, allow for this direct comparison to be made.

Because GF is a fixed factor that depends exclusively on the shape and size of the two explants that we want to compare, the same explants from different plants can be directly compared, provided that they have the same size and shape, and if regeneration occurs from the same tissue type(s).

The GCF allows different explants from the same plant (cultivar) to be compared, provided that all other experimental factors are the same, such as media, lighting, and so on, or allows comparison of different protocols for the same explant types.

Can conditions be optimized? In an absolute sense, conditions can be optimized for maximum output or RC for a specific explant under a defined set of in vitro conditions. In a relative sense, conditions can only be optimized when repeated by another scientist or laboratory in which all conditions are identical except for a single parameter, such as explant type or size.

Acknowledgments The first author thanks BioU (Tokushima, Japan) for providing all *Cymbidium* cultivars and to Prof. Seiichi Fukai for providing the chrysanthemum cultivar. The first author also wishes to thank Prof. Michio Tanaka for the use of research facilities.

Conflicts of interest The authors declare no conflicts of interest.

Glossary

A	Surface area of an explant
A_{epid}	Epidermal surface area of an explant
Conv	This subscript in the equations refers always to a conventional explant's parameters such as its surface (A_{conv}), volume (V_{conv}), or the number of organs it regenerates (SN_{conv})
Geometric factor (GF)	A unit that allows regeneration capacity to be calculated based on the size and shape of an explant. Its calculation also depends on the tissue from which regeneration occurs. It does not take into consideration the level of PGRs or other compounds or any other in vitro conditions that might influence organogenesis whose effect is considered to be null

Growth correction factor (GCF)	A proportional number expresses how many times more target organs can be regenerated from a source organ in a comparison of two explants. It is a theoretical value based on actual experimental data. The GCF applies exclusively to concrete organs (shoots, roots, leaves, flowers, PLBs, and so), but not to disorganized growth (callus)
k Factor	Is a proportional factor between GCF and GF that can be different depending on in vitro experimental conditions except for explant size and shape, which can affect the success of the regeneration process, such as medium, lighting, genotype, explant age, sampling time, etc. These factors are mathematically summarized as a *k* factor
Multipotency	The ability to derive organogenesis and regenerate any organ from any plant cell
n	The number of TCLs that can theoretically be prepared from a source explant
PLB %	Percentage of prepared explants that regenerate PLBs
R %	Percentage of prepared explants that regenerate organs
Regeneration capacity (RC)	The ability of an explant to form an organ or callus. The organogenic or morphogenic potential of an explant. RC takes into consideration both SN/PLB and *R* %
SN	Number of shoots that regenerate on an excised explant
SR %	Percentage of prepared explants that regenerate shoots
Totipotency	The ability to regenerate a whole plant from any plant cell
V	Volume of an explant

References

Caboni E, Tonelli M, Falasca G, Damiano C (1996) Factors affecting adventitious shoot regeneration in vitro in the apple rootstock 'Jork 9'. Adv Hortic Sci 10:146–150

Dobránszki J, Teixeira da Silva JA (2010) Micropropagation of apple—a review. Biotech Adv 28:462–488

Dobránszki J, Teixeira da Silva JA (2011) Adventitious shoot regeneration from leaf thin cell layers in apple. Sci Hortic 127:460–463

Dobránszki J, Teixeira da Silva JA (2013) In vitro shoot regeneration from transverse thin cell layers of apple leaves in response to various factors. J Hortic Sci Biotechnol 88:60–66

Dufour M (1990) Improving yield of adventitious shoots in apple. Acta Hortic 280:51–60

Ghnaya AB, Charles G, Hourmant A, Hamida JB, Branchard M (2007) Morphological and physiological characteristics of rapeseed plants regenerated in vitro from thin cell layers in response of zinc. CR Biol 330:728–734

James DJ, Passey AJ, Rugini E (1988) Factors affecting high frequency plant regeneration from apple leaf tissues cultured in vitro. J Plant Physiol 132:148–154

Kim KN, Ernst SG (1994) Effects of inhibitors on phenocritical events of in vitro shoot organogenesis in tobacco thin cell layer. Plant Sci 103:59–66

Magyar-Tábori K, Dobránszki J, Teixeira da Silva JA, Bulley SM, Hudák I (2010) The role of cytokinins in shoot organogenesis in apple. Plant Cell Tissue Organ Cult 101:251–267

Malabadi RB, Teixeira da Silva JA (2011) Thin cell layers: application to forestry biotechnology. In: Rao MNV, Soneji J (eds) Focus on tree micropropagation. Tree and forestry science and biotechnology, vol 5 (Special Issue 1). pp 14–18

Nhut DT, Teixeira da Silva JA, Le BV (2005) Thin cell layer sectioning for somatic embryogenesis in woody plants, chapter 4. In: Jain SM, Gupta PK (eds) Protocol for somatic embryogenesis in woody plants. forest sciences, vol 77. Kluwer Academic Publishers, Dordrecht, pp 577–585

Nhut DT, Hai NT, Don NT, Teixeira da Silva JA, Tran Thanh Van K (2006) Latest applications of thin cell layer (TCL) culture systems in plant regeneration and morphogenesis, chapter 60. In: Teixeira da Silva JA (ed) Floriculture, ornamental and plant biotechnology: advances and topical issues, vol II, 1st edn. Global Science Books, Ltd, Isleworth, pp 465–471

Pawlicki N, Welander M (1994) Adventitious shoot regeneration from leaf segments of in vitro cultured shoots of the apple rootstock Jork 9. J Hortic Sci 69:687–696

Teixeira da Silva JA (2003a) Thin cell layer technology in ornamental plant micropropagation and biotechnology. African J Biotechnol 2:683–691

Teixeira da Silva JA (2003b) Chrysanthemum: advances in tissue culture, postharvest technology, genetics and transgenic biotechnology. Biotechnol Adv 21:715–766

Teixeira da Silva JA (2004) Ornamental chrysanthemums: improvement by biotechnology. Plant Cell Tissue Organ Cult 79:1–18

Teixeira da Silva JA (2005a) Simple multiplication and effective genetic transformation (4 methods) of in vitro-grown tobacco by stem thin cell layers. Plant Sci 169:1046–1058

Teixeira da Silva JA (2005b) Effective and comprehensive chrysanthemum (*Dendranthema* X *grandiflora*) regeneration and transformation protocols. Biotechnology 4:94–107

Teixeira da Silva JA (2008) Plant thin cell layers: challenging the concept. Int J Plant Dev Biol 2:79–81

Teixeira da Silva JA (2010) Thin cell layers: power-tool for organogenesis of floricultural crops, chapter 32. In: Mohan Jain MS, Ochatt SJ (eds) Methods in molecular biology: protocols for in vitro propagation of ornamental plants, vol 589. Humana Press, Totowa, pp 377–391

Teixeira da Silva JA (2012) New basal media for protocorm-like body and callus induction of hybrid *Cymbidium*. J Fruit Ornam Plant Res 20:127–133

Teixeira da Silva JA (2013) The role of thin cell layers in regeneration and transformation in orchids. Plant Cell Tissue Organ Cult 113:149–161

Teixeira da Silva JA (2014) Novel factors affecting in vitro shoot culture of chrysanthemum (*Dendranthema x grandiflora* (Ramat.) Kitamura). Botanica Lithuanica (in press)

Teixeira da Silva JA, Dobránszki J (2011) The plant growth correction factor. I. The hypothetical and philosophical basis. Int J Plant Dev Biol 5:73–74

Teixeira da Silva JA, Dobránszki J (2013a) Plant thin cell layers: a 40-year celebration. J Plant Growth Regul 32:922–943

Teixeira da Silva JA, Dobránszki J (2013b) How timing of sampling can affect the outcome of the quantitative assessment of plant organogenesis. Sci Hortic 159:59–66

Teixeira da Silva JA, Tanaka M (2006) Multiple regeneration pathways via thin cell layers in hybrid *Cymbidium* (Orchidaceae). J Plant Growth Regul 25:203–210

Teixeira da Silva JA, Tanaka M (2010) Thin cell layers: the technique, chapter 2. In: Davey M, Anthony P (eds) Plant cell culture: methods express. Wiley-Blackwell, Chichester, pp 25–37

Teixeira da Silva JA, Giang DTT, Chan MT, Sanjaya Norikane A, Chai ML, Chico-Ruíz J, Penna S, Granström T, Tanaka M (2007a) The influence of different carbon sources, photohetero-, photoauto- and photomixotrophic conditions on protocorm-like body organogenesis and callus formation in thin cell layer culture of hybrid *Cymbidium* (Orchidaceae). Orchid Sci Biotechnol 1:15–23

Teixeira da Silva JA, Tran Thanh Van K, Biondi S, Nhut DT, Altamura MM (2007b) Thin cell layers: developmental building blocks in ornamental biotechnology. Floricult Ornam Biotechnol 1:1–13

Tran Thanh Van M (1973) In vitro control of de novo flower, bud, root and callus differentiation from excised epidermal tissues. Nature 246:44–45

Welander M (1988) Plant regeneration from leaf and stem segments of shoots raised in vitro from mature apple trees. J Plant Physiol 132:738–744