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Attempted genotype-independent induction of shoots in 15 carnation (*Dianthus caryophyllus*) cultivars from four explant types

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Abstract

Plant growth regulators were used in an attempt to directly induce adventitious shoots in 15 carnation (*Dianthus caryophyllus* L.; Caryophyllaceae) cultivars: 'Orange Sherbert', 'Avalanche', 'Magenta', 'La France', 'Stripe Red', 'Marie', 'Concerto PVP', 'Snap', 'Lucky Pierot', 'Cinnamon Tea', 'White Love', 'Siberia', 'Magesta', 'Spark Bruno', and 'Honono no Estejo'. After sowing seeds on top of autoclaved moistened filter paper, internodes between nodes 1 to 4 from the apical meristem were surface sterilized and induced to form shoots in the presence of 1 mg L^{-1} thidiazuron (TDZ) and 1 mg L^{-1} α-naphthaleneacetic acid (NAA) on basal Murashige and Skoog (MS) medium. Shoots were excised from mother explants and subcultured on Hyponex® (N-P-K = 6.5-6-19) medium containing 30% (w/v) sucrose to establish *in vitro* stock cultures, which was possible for all 15 cultivars. After two sub-cultures to eliminate the possible influence of seed-derived heterogeneity, internodes, transversal thin cell layers (tTCLs) from internode tissue, nodes and leaves from nodes 1 to 4 of one-month-old stock plantlets were excised and placed on MS basal medium containing 0, 1, 2, 4 or 8 g L^{-1} 2,4-D, TDZ, kinetin (Kin) or 6-benzyladenine (BA), together with 0, 0.5, or 1.0 mg L^{-1} NAA. Shoots formed best in the presence of 1 mg L^{-1} TDZ and 0.5 mg L^{-1} NAA from all cultivars. Callus, which was induced in the presence of BA, Kin and 2,4-dichlorophenoxyacetic acid, was not quantified. Results from all 15 cultivars were pooled: the largest number of shoots formed from nodes (4.85 per explant), although many more shoot initials formed. Internode tissue and tTCLs performed poorly. Individual shoots one-cm long excised from explants rooted easily (100%) in Hyponex® medium. This study provides a relatively effective and almost genotype-independent protocol for the establishment of carnation *in vitro* cultures.

Key words: carnation, Hyponex®, plant growth regulator, thin cell layer.
Abbreviations: 2,4-D, 2,4-dichlorophenoxyacetic acid; BA, 6-benzyladenine (syn. BAP, 6-benzylaminopurine); GCF, growth correction factor; IAA, indole-3-acetic acid; Kin, kinetin; NAA, α-naphthaleneacetic acid; PGR, plant growth regulator; SDDW, sterilized double-distilled water; TCL, thin cell layer; lTCL, longitudinal TCL; tTCL, transversal TCL; TDZ, *N*-phenyl-*N*'-1,2,3-thiadiazol-5-yl-urea or thidiazuron.

Introduction

Carnation (*Dianthus caryophyllus* L., Caryophyllaceae) is an economically important ornamental crop worldwide. The *in vitro* culture of carnation has been well studied and some key findings are summarized next. Frey and Janick (1991) found that petals of three cultivars were more responsive to shoot formation than nodes, internodes, or leaves in the presence of thidiazuron (*N*-phenyl-*N*'-1,2,3-thidiazol-5-yl urea; TDZ) and α-naphthaleneacetic acid (NAA). Casas et al. (2010) also found that a TDZ + NAA combination was essential for shoot formation from nodes, only TDZ from internodes (Watad et al. 1996), while 6-benzyladenine (BA) and NAA were required to induce shoots from leaves, a combination that was suitable for nodes, as found by Nontaswatsri and Fukai (2007), or for roots, as found by Seo et al. (2007). Gutiérrez-Miceli et al. (2010) found that NAA and BA stimulated callus while NAA, kinetin (Kin) and silver nitrate ($AgNO_3$) were needed to induce shoots from callus. Karami et al. (2007) induced callus from petals in the presence of BA and 2,4-dichlorophenoxyacetic acid (2,4-D), while shoots were induced from callus after transfer to picloram-supplemented medium. Jang et al. (2002) used a medium supplemented with Kin and indole-3-acetic acid (IAA) to induce shoots. Kanwar and Kumar (2009) found that a 2,4-D + BA combination induced most callus from leaf and internode explants, but shoot differentiation was only possible in the presence of TDZ and zeatin alone or in combination with NAA and/or IAA.

Thin cell layers (TCLs), which tend to be more sensitive to changes in the medium and medium constituents (Teixeira da Silva, Dobránszki 2013), were also used in this study. TCLs are broadly characterized as being transversal (when they traverse several tissue types; tTCLs) or longitudinal (when they are cut longitudinally through a single tissue type; lTCLs). The small size of these TCLs,

usually 1 to 2 mm thick and about 1 mm^2 in area, tend to make the explant ultra-sensitive to media, to changes in medium composition and to abiotic factors and stresses when applied in *in vitro* culture conditions, making them a very sensitive and thus ideal plant cell and tissue system for environment- and stress-related studies. Consequently, it is common to observe a single organogenic programme developing from a TCL in response to a particular set of *in vitro* conditions, and even though the actual number of organs tends to be considerably less than values obtained in a conventional explant, the true organogenic potential can be calculated by employing the Plant Growth Correction Factor (GCF; Teixeira da Silva, Dobránszki 2011; 2014). No other *in vitro* study on carnation, including the wide literature that tested the effect of PGRs, used TCL explants. Given the importance and versatility of this explant type in plant biotechnology, tTCLs were employed in this study, and their response to the treatments employed were contrasted to the response of three conventionally used explants, namely leaves, nodes and internodes.

This study attempted to establish a cultivar-independent protocol in order to achieve two main objectives: (i) to establish *in vitro* stocks from seed-derived and laboratory-grown plants; (ii) to induce shoots from different in vitro-derived tissues, specifically the use of three conventional explants (leaves, nodes, internodes) and a new explant type, not yet tested for any carnation cultivar, tTCLs.

Materials and methods

Chemicals and reagents

All PGRs were of the highest analytical grade available and were purchased from Sigma-Aldrich (St. Louis, USA). All other chemicals and reagents were purchased from either Wako Chemical Co. (Osaka, Japan) or Nacalai Tesque (Kyoto, Japan), the cheapest choice at the highest tissue-culture grade, unless specified otherwise.

Plant material and seeding conditions

Seeds of 15 carnation cultivars ('Orange Sherbert', 'Avalanche', 'Magenta', 'La France', 'Stripe Red', 'Marie', 'Concerto PVP', 'Snap', 'Lucky Pierot', 'Cinnamon Tea', 'White Love', 'Siberia', 'Magesta', 'Spark Bruno', and 'Honono no Estejo') were purchased from a Japanese online dealer. Seeds, some of which were very small, were soaked in between two layers of filter paper in 10-cm diameter glass Petri dishes overnight, then placed, 10 to 50 seeds per Petri dish, depending on the size of seeds, on top of two sheets of autoclaved, moistened filter paper (Advantec Toyo 2, 110 mm, Toyo Roshi Kaisha, Ltd., Tokyo, Japan). Prior to autoclaving, filter paper was trimmed to 90 mm circles to fit glass Petri dishes. Approx. 4 mL of sterilized double-distilled water (SDDW) was applied per Petri dish, and SDDW was added, as needed, to maintain the filter paper moist at all times. The seeds of all cultivars germinated within one week, and seed viability or percentage seed germination were not measured as these were not important aspects of this study. The external surface of Petri dishes was sterilized by swabbing with 80% (v/v) ethanol and Petri dishes were placed under environmental conditions identical to those employed in the in vitro experiments outlined next.

Explant sterilization and in vitro *establishment*

A modified protocol of Casas et al. (2010) was used for explant sterilization while a modified Nontaswatsri and Fukai (2007) protocol was used for shoot induction. Nodal segments 1 cm long from 6-cm long seedlings growing in Petri dishes were cut with sterile feather blades, gently rinsed in SDDW, rinsed three times in 70% (v/v) ethanol, surface sterilized for 8 min in 2% (w/v) sodium hypochlorite containing 0.01% (v/v) Tween-20, then rinse three times in SDDW. The ends of nodal segments were trimmed to obtain a section 6 mm long with the node (and axillary buds) in the middle. Nodes were plated on Murashige and Skoog (1962; MS) medium containing 1.0 mg L^{-1} TDZ, 0.1 mg L^{-1} NAA, 30 g L^{-1} sucrose, and 2 g L^{-1} Gellan gum (Gelzan®; CP Kelco Inc., J.M. Huber Corp.; GA, USA) for 10 days and then subcultured on the same medium (freshly made each time) every 10 days until shoots formed. Individual shoots (1 to 2 cm long) were then transferred to 500-mL glass bottles (AsONe, Osaka, Japan) holding MS medium containing 1.0 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 30 g L^{-1} sucrose and 2 g L^{-1} Gellan Gum (shoot induction medium, or SIM). Bottles were not ventilated and lids were closed off with air-permeable Parafilm M® (Bemis NA, Neenah, WI, USA). pH of all media was adjusted to 5.8 with 1 N NaOH or HCl prior to autoclaving at 100 KPa for 17 min. Cultures were placed on 25 mL medium in 9-cm diameter Petri dishes, closed off with air-permeable Parafilm M®, at 25°C, under a 16-h photoperiod with a light intensity of 45 μmol m^{-2} s^{-1} provided by plant growth fluorescent lamps (40 W; Homo Lux, Matsushita Electric Industrial Co., Japan).

Testing plant growth regulators for shoot induction, and rooting of shoots

To induce shoots, four explant types (internodes, tTCLs from internode tissue, nodes and leaves) from nodes 1 to 4 of 8-cm tall stock plantlets of all 15 cultivars were excised and placed on MS basal medium containing 0, 1, 2, 4 or 8 mg L^{-1} 2,4-D, TDZ, Kin or BA, together with 0, 0.5, or 1.0 mg L^{-1} NAA. Leaves (4 to 5 mm long) were used intact. Nodes (5 mm long) contained 1-mm of internode tissue on either extreme. Internode tissue was left intact, and each was 1 cm long. tTCLs were prepared by slicing internode tissue using autoclaved Feather blades (Product number: 99077; FEATHER Safety Razor Co., Ltd., Osaka, Japan) into concentric circles 1 mm thick and an area of 0.79 mm^2 (converted by πr^2). All tTCLs were prepared while explants were submerged under 20 mL of SDDW. Prior to plating, tTCLs were rinsed in a fresh dose of SDDW and blades were

replaced for each new internode. After initial trials, it was ascertained that all explants were most responsive to TDZ in the presence of NAA while 2,4-D, Kin and BA induced only callus, or no shoots. Thus, shoot formation was only quantified for the TDZ+NAA combination. All cultures were placed under abiotic growth conditions specified for *in vitro* culture establishment. Callus induction was not assessed as callus formation was not a desirable organogenic outcome that could lead to somaclonal variation. Shoots one-cm long that formed from any explant derived from any treatment were transferred to 50 mL of Hyponex® Plant Food medium (N-P-K = 6.5-6-19; Hyponex Japan Corp. Ltd, Osaka, Japan) containing 30 g L^{-1} sucrose held in 500-mL bottles, at 10 shoots per bottle.

Statistical analyses

Experiments were organized according to a randomized complete block design with three blocks of 10 replicates per treatment. All experiments were repeated in triplicate (n = 30, total sample size per treatment). Data was subjected to analysis of variance (ANOVA) with mean separation by Duncan's multiple range test using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

Based on the literature, it was broadly shown that TDZ and/or BA, alone or in combination with NAA, could induce shoots without intermediate callus formation, although the same cultivars are able to induce callus in the presence of other PGRs (Teixeira da Silva 2014b). In this study, preliminary trials using four explant types (internodes, tTCLs from internode tissue, nodes and leaves) revealed that callus was induced in the presence of BA, 2,4-D and Kin. Since callus-derived shoot formation is an undesirable result for clonal propagation, direct shoot formation, a key objective of this study, in the presence of a combination of TDZ and NAA was tweaked for all 15 cultivars. Callus is however desirable when trying to induce somaclonal variants that can then be used to screen for new cultivars with novel characteristics that would fortify the ornamental value of this plant, such as leaf ornamentation, novel flower colours or resistance to abiotic stresses. Consequently, using seed-derived plantlets from a wide range of cultivars, the objective was to induce direct shoot formation without the intermediate formation of callus. Doing so would provide a practically useful clonal propagation protocol.

Seed-derived plantlets could be derived from the seeds of all 15 cultivars and stock mother plants. From there the four experimental explant types were derived, which could be effectively clonally propagated. Three explants tested in this study are the most commonly used in the literature, namely leaves, nodes and internodes. Yet, to date, no study has examined the use of TCLs. In this study, tTCLS were also used to assess whether a higher level of organogenesis could be achieved. This is because, most likely because of its reduced size and thus enhanced sensitivity, organogenesis from TCLs can often result in more organ-specific organogenesis, as has been observed in quite a few classes of crops (Teixeira da Silva, Dobránszki 2013; 2014) and orchids (Teixeira da Silva 2014a).

The absence of either TDZ or NAA, as well as the use of a high concentration of either, alone or combination, resulted in no or poor shoot formation (Table 1). TDZ at 1 or 2 mg L^{-1} in combination with 0.5 mg L^{-1} NAA was the most responsive set of PGR combinations for all explant types, as observed in 'Stripe Red' (Fig. 1A, B). Most shoots formed from internodes (4.85 per explant), although a conversion of shoot number from tTCLs following application of the GCF (Teixeira da Silva, Dobránszki 2011; 2014) suggests that 10.9 shoots would form if the same area of explant tissue were used (equivalent to 1 cm of internode tissue). Thus, at face value, the actual number of shoots that formed from tTCLs was actually very low (1.09 per explant; Table 1), but the potential regeneration ability, as expressed by the GCF, was superior to leaves, nodes and internodes. Only large-scale experiments that test several dozen or hundred explants would be able to confirm this fact. Leaves and tTCLs were most sensitive to PGRs – as assessed by greater mortality and/or lack of organogenesis – while internodes, followed by nodes, were the most responsive – as assessed by wider range of treatments leading to caulogenesis (shoot formation; Table 1). Transfer of shoots to BA+NAA-containing MS medium resulted in stable shoot formation over at least two sub-cultures, and served as a stable source of explant stock.

The use of shoots is the most viable way to clonally propagate carnation and other ornamentals. There is much literature on shoot formation in carnation and only some representative results are presented here as a rough form of comparison, since protocols and cultivars differ considerably, making direct comparisons literally impossible. Frey and Janick (1991) found that a mixture of TDZ and NAA resulted in shoot formation from never more than 67% of leaf explants, or 10 to 20% in nodes and internodes, but in those three explants only very few combinations resulted in organogenesis; however, almost all combinations of different concentrations of NAA and TDZ resulted in shoot formation from petals. In contrast, in this study, most NAA+TDZ combinations resulted in shoot formation from internodes, but least from tTCLs (Table 1). Nontaswatsri et al. (2002) found that an initial 10-day impulse with TDZ followed by culture in 1 mg L^{-1} BA resulted in direct shoot formation in 35 standard and spray carnation cultivars. Inspired by the widely cultivar-independent nature of that protocol, a similar two-step strategy was used in this study: an initial TDZ+NAA pulse for 10 days was followed by culture on SIM, which contained BA+NAA. Seo et al. (2007) found that 8.16 shoots could

Table 1. Effect of plant growth regulators on shoot development from four explant types averaged over 15 carnation cultivars. Explants were derived from stock plantlets that were induced in the presence of 1 mg L^{-1} TDZ and 1 mg L^{-1} NAA then transferred to 1.0 mg L^{-1} BA and 0.1 mg L^{-1} NAA on basal MS medium, then rooted on Hyponex® medium containing 30 g L^{-1} sucrose (Fig. 1C). Although many more shoot initals formed, these were not quantified and only expanded and developed shoots were used for quantification (because they are practically useful). N = 20 × three replicates (pooled for 15 cultivars). Different letters within a column indicate significant differences ($P \leq 0.05$) according to Duncan's multiple range test. Use of the Plant Growth Correction Factor (Teixeira da Silva, Dobránszki 2011; 2014) would hypothetically result in a precisely 10-fold increase in the number of shoots per explant (since 10 tTCLs can be prepared from one 1-cm long internode explant)

PGR treatment	Explant type			
(mg L^{-1})	Leaf	Node	Internode	tTCL
TDZ (0) + NAA (0)	0 c	0 e	0.14 fg	0 c
TDZ (0) + NAA (0.5)	0 c	0.17 d	0.23 fg	0 c
TDZ (0) + NAA (1)	0 c	0.09 de	0.06 g	0 c
TDZ (1) + NAA (0)	0 c	0.26 d	0.68 f	0 c
TDZ (1) + NAA (0.5)	1.18 a	2.43 b	4.85 a	1.09 a
TDZ (1) + NAA (1)	0.29 b	1.24 c	2.16 c	0 c
TDZ (2) + NAA (0)	0 c	0 e	0.49 f	0 c
TDZ (2) + NAA (0.5)	0.98 a	3.03 a	3.81 b	0.88 a
TDZ (2) + NAA (1)	0 c	2.79 ab	1.73 d	0 c
TDZ (4) + NAA (0)	0 c	0 e	0 g	0 c
TDZ (4) + NAA (0.5)	0.63 ab	1.03 c	1.98 cd	0.24 b
TDZ (4) + NAA (1)	0 c	0.06 de	1.16 e	0 c
TDZ (8) + NAA (0)	0 c	0 e	0 g	0 c
TDZ (8) + NAA (0.5)	0 c	0.32 d	0.21 fg	0 c
TDZ (8) + NAA (1)	0 c	0 e	0 g	0 c

regenerate from each leaf explant in the presence of 2.5 μM of BA. Jang et al. (2002) found that as many as 26.25 shoots could regenerate from each explant (exact explant – source and size – unclear) in the presence of IAA and kinetin. Kanwar and Kumar (2009) produced most shoots (from about 21% of callus) from a TDZ+IAA or zeatin+IAA combination, equally successfully from leaves or from internodes, but regeneration was indirect, i.e., through a callus phase. In this study, callus was very rudimentary, or did not form at all, for example from tTCLs, and it was assumed that shoot formation took place from tissue other than callus, although detailed histological analyses would be required to confirm this fact.

Shoots were successfully rooted on Hyponex® medium containing 30 g L^{-1} sucrose (Fig. 1C), and a profuse root system formed for all cultivars. The apparent appearance of hyperhydricity that typified shoots cultures after 45 days (Fig. 1B) disappeared after transfer to Hyponex® medium. Casas et al. (2010) provide practical protocols for reducing or eliminating hyperhydricity from carnation

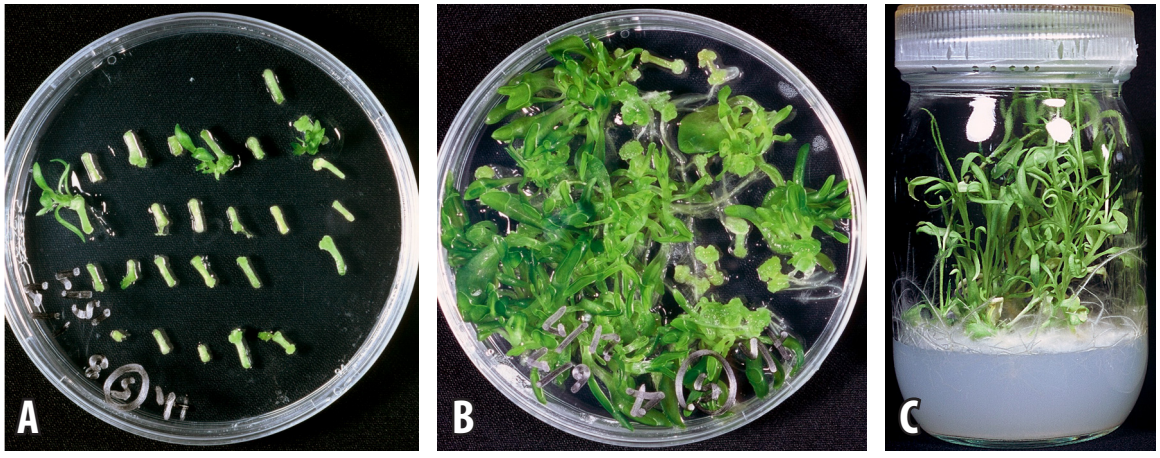

Fig. 1. The response of carnation (*Dianthus caryophyllus* L.) 'Stripe Red' internodes to optimized shoot induction medium (1.0 mg L^{-1} BA, 0.1 mg L^{-1} NAA, 20 g L^{-1} sucrose and 2 g L^{-1} Gellan gum) after 25 days (A) and 45 days (B). Transfer to Hyponex® medium containing 30 g L^{-1} sucrose resulted in profuse rooting and healthy vigorous plantlet formation in one month (C).

shoot cultures. Although plantlets were not acclimatized in this study, the lack of hyperhydricity and a strong and profuse root system bode well for the next step of the micropropagation protocol, which still need to be explored.

The carnation literature reports several dozen papers on the regeneration of many cultivars from different explant sources, using a wide range of PGRs. This study confirms that TDZ and NAA are a suitable combination for direct shoot formation, and provides an almost genotype-independent protocol. Moreover, shoot formation from internode-derived tTCLs indicates that this explant could serve as a suitable explant for mass production of shoots and clonal propagation when the GCF is factored in.

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References

Casas J.L., Olmos E., Piqueras A. 2010. *In vitro* propagation of carnation (*Dianthus caryophyllus* L.). In: *Protocols for* inVitro *Propagation of Ornamental Plants*. Methods in Molecular Biology, Vol. 589. Springer, Berlin, pp. 109–116.

Frey L., Janick J. 1991. Organogenesis in carnation. *J. Am. Soc. Hortic. Sci.* 116: 1108–1112.

Gutiérrez-Miceli F.A., Arias L., Juarez-Rodríguez N., Abud-Archila M., Amaro-Reyes A., Dendooven L. 2010. Optimization of growth regulators and silver nitrate for micropropagation of *Dianthus caryophyllus* L. with the aid of a response surface experimental design. *In Vitro Cell. Dev. Biol. Plant* 46: 57–63.

Jang S.J., Choi Y.J., Park K.Y. 2007. Effects of polyamines on shoot and root development in Arabidopsis seedlings and carnation cultures. *J. Plant Biol*. 45: 230–236.

Kanwar J.K., Kumar S. 2009. Influence of growth regulators and explants on shoot regeneration in carnation. *Hort. Sci.* (Prague) 36: 140–146.

Karami O., Deljou A., Mahmodi Pour A. 2007. Repetitive somatic embryogenesis in carnation on picloram supplemented media. *Plant Growth Reg.* 51: 33–39.

Murashige T., Skoog F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue culture. *Physiol. Plant*. 15: 473–497.

Nontaswatsri C., Fukai S. 2007. Carnation (*Dianthus caryophylus* L.). In: Agrobacterium *Protocols*, Vol. 2. Methods in Molecular Biology, Vol. 344. Springer, Berlin, pp. 311–320.

Nontaswatsri C., Fukai S., Touma T., Goi M. 2002. Comparison of adventitious shoot formation from node and leaf explants of various carnation (*Dianthus caryophyllus* L.) cultivars. *J. Hortic. Sci. Biotechnol.* 77: 520–525.

Seo J.W., Kim S.W., Min S.R., Liu J.R. 2007. High frequency somatic embryogenesis and plant regeneration in root explant cultures of carnation. *Plant Biotechnol. Rep.* 1: 67–70.

Teixeira da Silva J.A. 2012. Is BA (6-benzyladenine) BAP (6-benzylaminopurine)? *Asian Austral. J. Plant Sci. Biotechnol.* 6: 121–124.

Teixeira da Silva J.A. 2014a. The role of thin cell layers in regeneration and transformation in orchids. *Plant Cell Tissue Organ Cult.* 113: 149–161.

Teixeira da Silva J.A. 2014b. Callus induction from 15 carnation (*Dianthus caryophyllus* L.) cultivars. *J. Plant Dev.* /in press/

Teixeira da Silva J.A., 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 J.A., Dobránszki J. 2013. Plant thin cell layers: a 40-year celebration. *J. Plant Growth Reg.* 32: 922–943.

Teixeira da Silva J.A., Dobránszki J. 2014. Dissecting the concept of the thin cell layer: theoretical basis and practical application of the Plant Growth Correction Factor to apple, *Cymbidium* and chrysanthemum. *J. Plant Growth Reg.* 33: 881–895.

Watad A.A., Ahroni A., Zuker A., Shejtman H., Nissim A., Vainstein A. 1996. Adventitious shoot formation from carnation stem segments: a comparison of different culture procedures. *Sci. Hortic.* 65: 313–320.

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