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CALLUS INDUCTION FROM 15 CARNATION (*DIANTHUS CARYOPHYLLUS* L.) CULTIVARS

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Abstract: Plant growth regulators (PGRs) were used to induce callus 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. Seeds were initially sown on autoclaved moistened filter paper and internodes of surface-sterilized seedlings were used as explants. Most callus was induced in the presence of 0.5 mg/L α-naphthaleneacetic acid used together with 1 mg/L 6-benzyladenine or 1 mg/L 2,4-dichlorophenoxyacetic acid on basal Murashige and Skoog medium. Callus is not a desirable method to clonally propagate important germplasm but can serve as one possible way of deriving periclinal mutants as a result of somaclonal variation.

Keywords: carnation, internode, plant growth regulator, thin cell layer

Introduction

The induction of friable and/or embryogenic callus from carnation (*Dianthus caryophyllus* L., family Caryophyllaceae), an economically important ornamental, has been well studied. α-Naphthaleneacetic acid (NAA) and 6-benzyladenine (BA) [GUTIÉRREZ-MICELI & al. 2010] or BA and 2,4-dichlorophenoxyacetic acid (2,4-D) [KARAMI & al. 2007] were able to stimulate callus formation. KANWAR & KUMAR (2009) also found that a combination of 2,4-D + BA was most efficient for the induction of callus, primarily from leaf and internode explants. In all these studies, shoots were then induced to form from callus in the presence of other media and plant growth regulators (PGRs). A previous study [TEIXEIRA DA SILVA, 2014] found that shoots could be regenerated directly on MURASHIGE & SKOOG (MS; 1962) medium from the same 15 cultivars tested in this study using different explants (nodes, internodes, leaves, thin cell layers (TCLs)), although each explant showed a wide range of variation depending on the explant and PGR used. TCLs are important explants as they allow for a greater fine-scale control of plant organogenesis [TEIXEIRA DA SILVA & DOBRÁNSZKI, 2013]. In the TEIXEIRA DA SILVA (2014) study, it was found that internodes were most responsive *in vitro*, and were thus used as the explant for this study on callus induction.

The objective of this study was to induce organogenic or embryogenic callus from 15 as-yet unexplored carnation cultivars. Although callus is generally not a desired pathway for carnation or even for other ornamentals, since somaclonal variation can occur, it can

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also have positive benefits, including the discovery of new mutants with novel growth form, flower colour or other ornamentally attractive growth characteristics.

Materials and methods

The materials and methods related to seeding, explant sterilization and *in vitro* establishment strictly follow the protocols outlined by TEIXEIRA DA SILVA (2014). 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

As described in TEIXEIRA DA SILVA (2014), "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, 10-50 seeds/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. Approximately 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 & al. (2010) was used for explant sterilization, as described in TEIXEIRA DA SILVA (2014): "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 rinsed 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 MS medium with 1.0 mg/L thidiazuron (TDZ), 0.1 mg/L NAA, 30 g/L sucrose, and 2 g/L Gellan gum (Gelzan®; CP Kelco Inc., J.M. Huber Corp.; GA, USA) for 10 d and then subcultured on the same freshly made medium every 10 days until shoots formed. Individual shoots (1-2 cm long) were then transferred to 500 mL glass bottles (AsONe, Osaka, Japan) holding MS medium with 1.0 mg/L BA, 0.1 mg/L NAA, 30 g/L sucrose and 2 g/L 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 provided by plant growth fluorescent lamps (40 W; Homo Lux, Matsushita Electric Industrial Co., Japan)". These shoots served as stock material for the callus-induction experiments.

Testing plant growth regulators for callus induction

To induce callus, 1 cm long internodes from nodes 1-4 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 g/L 2,4-D, Kin or BA (found to be the most responsive PGRs in the wider carnation tissue culture literature), together with 0, 0.5 or 1.0 mg/L NAA. Following initial trials, it was ascertained that explants were most responsive to 2,4-D or BA in the presence of NAA. Thus, callus formation was only quantified for the 2,4-D/BA+NAA combination. All callus cultures were placed under growth conditions specified for *in vitro* culture establishment.

Statistical analyses

Experiments were organized according to a randomized complete block design (RCBD) 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 (DMRT) using SAS® vers. 6.12 (SAS Institute, Cary, NC, USA). Significant differences between means were assumed at $P \leq 0.05$.

Results and discussion

The direct formation of shoots from node and leaf explants in carnation is generally genotype-dependent, although the use of TDZ tends to produce shoots without an intermediate callus phase [NONTASWATSRI & al. 2002]. In contrast, callus is generally not a desired organogenic route in clonal propagation since callus-derived shoots can result in somaclonal variation and thus phenotypic differences. However, somaclonal variation can be an important result for ornamentals like carnation since alternative phenotypic characters may be derived, such as novel flower colours, or leaf forms, all with ornamental and thus economic potential. The carnation literature reveals a broad trend: TDZ and/or BA, alone or in combination with NAA, can be used to induce shoots without intermediate callus formation (e.g., NONTASWATSRI & al. 2002; CASAS & al. 2010; VARSHNEY & al. 2013). However, for the 15 cultivars tested in this study, the absence of TDZ or NAA, as well as the use of a high concentration of either, alone or combination, resulted in no or poor shoot formation [TEIXEIRA DA SILVA, 2014]. Thus, in this case, where a direct route for shoot formation was not possible, an alternative, indirect route, through friable and organogenic callus, was tested in this study.

Magenta was the most responsive cultivar to callus formation, followed by Snap, while Le France was least responsive to all combinations of NAA + BA/2,4-D (Fig. 1). In this study, the use of 0.5 mg/L NAA together with 1 mg/L BA or 1 mg/L 2,4-D on basal MS medium induced most callus across cultivars (Fig. 1), although the visual aspect of callus from most cultivars was similar (friable, green or white) after 45 days, with limited shoot formation (Fig. 2). Interestingly, *Dianthus ciliatus* ssp. *dalmaticus* and *D. giganteus*

ssp. *croaticus* formed both green organogenic callus in which shoot initials were embedded, as well as white embryogenic callus that contained embryo-like structures when seedling-derived nodal segments were placed in MS medium supplemented with 2,4-D, BA and NAA [RADOJEVIĆ & al. 2010].

Callus induction in carnation tissue cultures is widely reported in the literature but only a few representative studies are listed here. FREY & JANICK (1991) found that TDZ and NAA resulted in callus formation from 'Scania', 'Improved White Sire' and 'Sandra' petal explants but the same three cultivars formed embryogenic callus from internodes in the presence of 2,4-D [FREY & al. 1992]. SEO & al. (2007) found that the same combination of PGRs resulted in callus induction from root explants. KANWAR & KUMAR (2009) produced most callus in 'Indios' from a 2,4-D+BA combination from leaves or internodes, and shoots could then be regenerated from callus. A subsequent study by the same authors [KANWAR & al. 2010] indicated that the use of TDZ in combination with indole-3-acetic acid could result in the most shoots from leaf- and internode-derived callus in 'Tempo'. White and green friable callus, as was observed in this study, was also induced from root segments of 'Grenadin' in the presence of a wide range of concentrations (0-3.0 mg/L) of NAA and BA [YAACOB & al. 2013], and also from internode explants in the presence of 2,4-D for an unspecified cultivar [ARIF & al. 2014].

Even though cultures in this study were not ventilated, no visible hyperhydricity was observed. This was not the case in a study by MOHAMED (2011), who employed *D. caryophyllus* 'White Sim' nodal explants to induce shoots directly in the presence of BA on MS medium when culture flasks were aerated, although in that study, both callus formation and hyperhydricity were observed in non-aerated vessels, suggesting that aeration, or the lack of it, can alter the organogenic state of explants in the presence of the same PGRs [CASAS & al. 2010], possibly as a result of the action of ethylene, which can build up in culture flasks.

Conclusions

In this study, friable and organogenic callus could be induced from 15 carnation cultivars in the presence of six combinations of NAA, BA and 2,4-D. Most prolific callus in all 15 cultivars was in the presence of 0.5 mg/L NAA, 1.0 mg/L BA and 1.0 mg/L 2,4-D, as assessed by callus fresh weight. Although not the most desirable route for the clonal propagation of plants through shoot induction, the induction of shoots via an indirect callus route can be beneficial where direct shoot induction is not possible, as was observed in several of the same cultivars in separate experiments [TEIXEIRA DA SILVA, 2014]. This study provides an additional and expanded set of data to support callus induction in unexplored or difficult-to-propagate carnation cultivars or *Dianthus* species. VARSHNEY & al. (2013) used biochemical and molecular marker-based analyses to verify the genetic stability of tissue culture-derived shoots. YAACOB & al. (2013) employed cytological analyses to confirm the cytological stability of regenerants. Such methods of verification are recommended for all carnation tissue culture studies.

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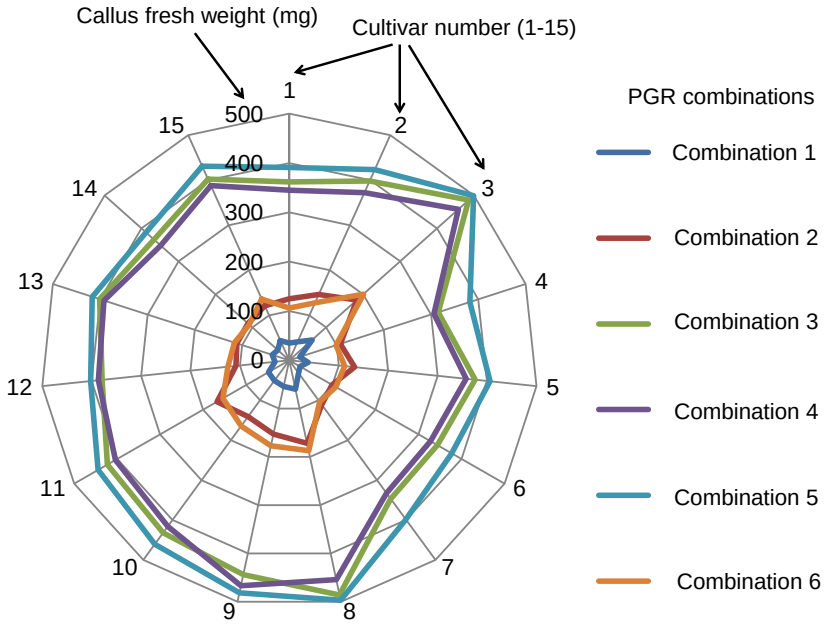

Fig. 1. The response (fresh weigh (mg) of callus) of 15 carnation (*Dianthus caryophyllus* L.) cultivar internodes to multiple combinations and concentrations (mg/L) of NAA, BA and 2,4-D on basal MS medium after 25 days. Cultivars: 1 = Orange Sherbert; 2 = Avalanche; 3 = Magenta; 4 = La France; 5 = Stripe Red; 6 = Marie; 7 = Concerto PVP; 8 = Snap; 9 = Lucky Pierot; 10 = Cinnamon Tea; 11 = White Love; 12 = Siberia; 13 = Magesta; 14 = Spark Bruno; 15 = Honono no Estejo. Plant growth regulator (PGR) combinations (in mg/L; NAA, BA, 2,4-D): combination 1 (0, 0, 0); combination 2 (0.5, 0.5, 0.5); combination 3 (0.5, 0, 1.0); combination 4 (0.5, 1.0, 0); combination 5 (0.5, 1.0, 1.0); combination 6 (1.0, 1.0, 1.0).

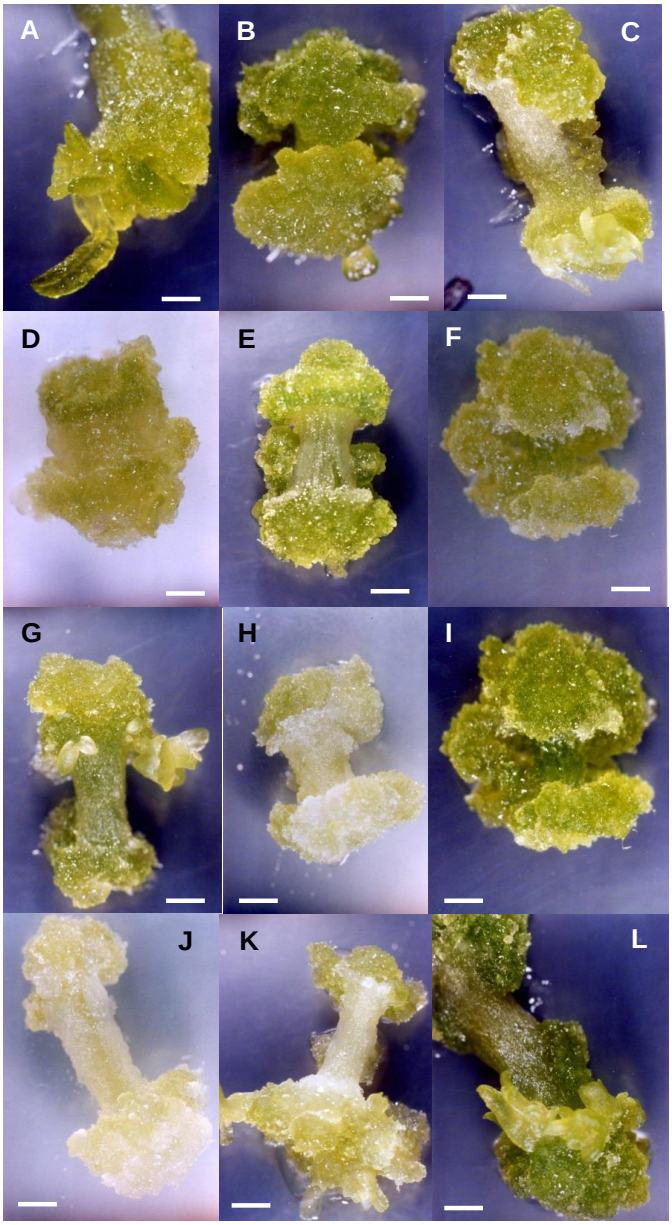

Fig. 2. The response of 12 carnation (*Dianthus caryophyllus* L.) cultivar internodes (Magenta, Snap and Spark Bruno not represented) to 0.5 mg/L NAA together with 1 mg/L BA or 1 mg/L 2,4-D on basal MS medium after 25 days. (A) Avalanche; (B) Cinnamon Tea; (C) Concerto PVP; (D) Honono no Estejo; (E) La France; (F) Lucky Pierot; (G) Magesta; (H) Marie; (I) Orange Sherbert; (J) Siberia; (K) Stripe Red; (L) White Love. Bars = 1 mm.

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