In vitro Propagation and Plantlet Regeneration from *Doritaenopsis* Purple Gem ‘Ching Hua’ Flower Explants

Wagner A. Vendrame¹ and Ian Maguire

Tropical Research and Education Center, University of Florida, 18905 SW 280th St., Homestead, FL 33031-3314

Virginia S. Carvalho

Departamento de Fitotecnica, Universidade Federal de Viçosa, Viçosa, MG, 36570-000, Brazil

Additional index words. micropropagation, Orchidaceae, orchids, orchid hybrids, TDZ, NAA

Abstract. The effects of four types of explants removed from 10-cm flower stalks of *Doritaenopsis* Purple Gem ‘Ching Hua’ (immature apical flower buds, immature lateral flower buds, flower stem nodes, and flower pedicel sections) and combinations of two plant growth regulators [naphthalene acetic acid (NAA) and thidiazuron (TDZ)] on direct in-vitro shoot induction and multiplication were studied. Immature apical flower buds were the only explants that showed induction and multiplication of shoots in vitro. NAA at 5.4 and 10.7 μM combined with either 4.5 or 9.1 μM TDZ provided the fastest and greatest percentages of shoot induction (27% to 40%) and the greatest numbers of shoot multiplication (111–160 shoots per explant). In vitro-induced shoots were rooted on medium containing 5.4 μM NAA and developed into plantlets with normal vegetative and reproductive morphology. Regenerated plantlets were acclimatized, showing 100% survival and establishment in greenhouse. Plantlets were grown to maturity and showed normal flower morphology. No floral off-types were observed. The high rates of shoot multiplication obtained offer a means for mass clonal propagation of this and possibly other related *Doritaenopsis* hybrids.

The production of new and improved orchid hybrids has focused mostly on new flower colors, color patterns, and flower size and number. Over 100,000 commercial orchid hybrids have been registered worldwide to date for either the cut flower or potted plant market. The rapid expansion of the market has increased the interest of growers and customers for new and improved orchids. By 2005, the demand for orchids had placed them in second place among potted floriculture crops in the United States with wholesale revenues estimated at USD 144 million (USDA, 2006). Furthermore, commercial orchid production currently represents 8% of global floriculture trade, due to the increased demand for orchid cut flowers (Martin and Madassery, 2006).

Doritaenopsis Guillaum. & Lami is a popular hybrid between *Phalaenopsis* Blume and *Doritis pulcherrima* Lindl. and is used as a potted plant or cut flower (Tsukazaki et al., 2000). General micropropagation procedures for *Doritaenopsis* and *Phalaenopsis* have been published (Arditti and Ernst, 1993).

Particularly for *Doritaenopsis*, in vitro propagation protocols have been developed using different explants and various concentrations of plant growth regulators. Explants used include flower stalk internodes (Lin, 1986), shoot tips of flower stalk buds (Tokuhara and Mii, 1993), dormant buds (Ernst, 1994), and root tip cultures (Park et al., 2003). Plant growth regulators used include benzyladenine (BA) (Lin, 1986), naphthalene acetic acid (NAA) combined with 6-benzylaminopurine (BAP) (Tokuhara and Mii, 1993), thidiazuron (TDZ) (Ernst, 1994), and zeatin (Park et al., 2003). Most of the studies performed to date involve the production of shoots through the formation of protocorm-like bodies (PLBs). Although PLBs can be produced from almost any part of the plant, callus formation may occur (Arditti and Ernst, 1993; Park et al., 2002), and PLBs tend to grow and develop more slowly than does direct shoot formation.

In preliminary studies we observed that various concentrations of TDZ combined with various concentrations of NAA were able to quickly induce direct shoot formation and high multiplication rates when using specific flower explants from *Phalaenopsis* and *Doritaenopsis* hybrids (W. Vendrame, unpublished data). To confirm those preliminary results, we repeated the experiment to verify the effects of four explant sources and combinations of two plant growth regulators (NAA and TDZ) at different concentrations

on direct in-vitro shoot induction and multiplication in cultures of *Doritaenopsis*. Plant regeneration, acclimatization, survival, and establishment were also evaluated.

Materials And Methods

Plant material and explant sterilization.

Plants of *Doritaenopsis* Purple Gem ‘Ching Hua’ were obtained from McCurdy’s Nursery (Homestead, FL). Four types of explants were removed from 10-cm flower stalks (Fig. 1A) from 4 to 7 Oct. 2004: 5.0-mm-long immature apical flower buds (AB), 5.0-mm-long immature lateral flower buds (LB), 10.0- to 20.0-mm-long flower stem nodes (SN), and 5.0- to 10.0-mm-long flower pedicel sections (PS). After removal from flower stalks, explants were washed in distilled water and surface-sterilized with 1% Alconox (Alconox, New York, NY) solution followed by two rinses in distilled water. Explants were sterilized as follows: 95% ethanol for 1 min, 1.5% sodium hypochlorite for 20 min under vacuum and with addition of Tween 20 (3 drops/100 mL solution), and three rinses in sterilized distilled water. Explants were maintained in sterilized distilled water before initiation of in-vitro cultures.

In vitro shoot induction and multiplication.

Explants were placed in 95 × 15 mm disposable plastic petri dishes containing a culture medium composed of Gamborg’s B5 major salts (Gamborg et al., 1968), MS minor salts (Murashige and Skoog, 1962), and C1 vitamins (Tisserat and Jones, 1999) supplemented with 20 g·L⁻¹ sucrose, 100 mg·L⁻¹ myo-inositol, 100 mg·L⁻¹ casein hydrolysate, 200 mL coconut water, and solidified with 6 g·L⁻¹ Bacto agar (Difco Laboratories, Sparks, MD). The medium pH was adjusted with either 0.1 N HCl or 0.1 N NaOH to 5.7 before autoclaving at 121 °C for 15 min at 1.2 kg·cm⁻². Multiplication treatments consisted of the same medium described above but supplemented with NAA at 5.4 or 10.7 μM combined with TDZ at 2.3, 4.5, or 9.1 μM . Cultures were maintained at 27 °C in the dark for 15 d for shoot induction followed by 150 d under an 18-h photoperiod provided by soft-white fluorescent lamps (4 × 9 A Philips, Philips Lighting Co., Somerset, NJ) that delivered 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF outside the containers for shoot multiplication.

Rooting and plantlet regeneration. Following induction and multiplication, in vitro-induced shoots were transplanted into Phytotech P700 culture boxes (Phytotechnology Laboratories, Shawnee Mission, KS) containing the same medium as described above for induction supplemented with 5.4 μM NAA. Cultures were maintained in a Percival E30B incubator (Percival Scientific, Perry, IA) at 27 ± 2 °C; an 18-h photoperiod provided by soft-white fluorescent lamps (6 × 9A Philips, Philips Lighting Co.) that delivered 80 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF outside the containers for root development and plantlet regeneration. Cultures were monitored weekly for root induction. Fully regenerated plantlets exhibiting well-developed roots and shoots measuring on average 5.0–6.0 cm long

Received for publication 15 Feb. 2007. Accepted for publication 18 Apr. 2007.

We thank Dave Baskin for help in obtaining the plant material for this study.

¹To whom reprint requests should be addressed; e-mail vendrame@ufl.edu

Fig. 1. In vitro propagation and plantlet regeneration from *Doritaenopsis* Purple Gem 'Ching Hua' flower explants. (A) Four types of explants removed from flower stalks are shown: 5.0-mm-long immature apical flower buds (AB), 5.0-mm-long immature lateral flower buds (LB), 10.0- to 20.0-mm-long flower stem nodes (SN), and 5.0- to 10.0-mm-long flower pedicel sections (PS) (bar = 10.0 mm). (B) Multiple shoot production (bar = 10.0 mm). (C) General in vitro developmental stages from unrooted (left) to rooted (right) shoots (bar = 10.0 mm). (D) Fully developed shoot showing well-developed roots (bar = 100 mm). (E) Regenerated plantlet in bloom.

(from root tip to leaf tip) were acclimatized and transferred to greenhouse after 120 d. Plantlets were transplanted to 10-cm pots containing 35% raw fir bark, 15% raw pine bark, 15% coconut fiber (Coco Gro-Brick, OFE International, Miami, FL) 15% charcoal, and 20% perlite, and horticultural foam as the substrate. Pots were maintained in greenhouse at average temperatures of 27 °C high/19 °C low and an average 220 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PPF. Pots were irrigated every other day with a solution of Peters Orchid Food (Spectrum Group, St. Louis) consisting of 30% total N, 10% P_2O_5 , 10% K_2O , 0.5% Mg, 0.02% B, 0.05% chelated Cu, 0.1% Fe,

0.05% Mn, 0.0005% Mo, and 0.05% Zn. Plants were maintained in greenhouse for 8–10 months when flowering was observed.

Experimental design and data analysis. For each explant type (immature apical flower buds, immature lateral flower buds, flower stalk nodal sections, and flower pedicel sections), 10 replications were used per treatment, each containing three or four explants. Treatments consisted of combinations of three concentrations of NAA with four concentrations of TDZ. For each treatment, induction percentage at 30 d and mean fresh weight of shoots and mean total number of shoots at 150 d were recorded. Induction

percentage was calculated based on the number of single explants showing direct shoot formation divided by the total number of explants in each treatment. For percent shoot induction, data were transformed using arcsin transformation before statistical analysis. Experiments were conducted in a completely randomized design and repeated. Analysis of variance (ANOVA; $P \leq 0.05$) was performed, and treatment means were compared using Duncan's multiple range test ($P \leq 0.05$) in the SAS program (SAS Institute, 1989).

Results And Discussion

In vitro shoot induction and multiplication.

Immature lateral flower buds, flower stem nodes, and flower pedicel sections failed to establish in vitro and did not exhibit induction response after 15 d, with subsequent necrosis and death of tissue, or >40% contamination after 30 d. Because contamination was observed after 30 d of culture initiation and on non-responsive cultures, contamination had no direct effect on the failure of cultures to establish but instead was a consequence of tissue decline over time. Immature apical flower buds were the only explants to exhibit induction response, as observed by swelling and enlargement of immature buds 15–30 d after culture initiation, and subsequent direct shoot formation (data not shown). Consequently, treatments were compared for the effects of plant growth regulators on induction and multiplication from AB explants.

In over 50% of in-vitro-initiated cultures from AB explants, the presence of phenolics was observed. Phenolics have been reported to oxidize in the culture medium and become phytotoxic to explants, thus requiring frequent subculturing to fresh medium (Morel, 1974; Ernst, 1994). Specifically in orchids, oxidation of phenolics was reported to account for browning or blackening of explants followed by death (Flamee and Boesman, 1977; Morel, 1974). However, in preliminary studies we observed that the presence of phenolics did not affect the induction, multiplication, and subsequent rooting of shoots developed (W. Vendrame, unpublished data). In the present study, no inhibitory effects by phenolics on shoot induction or multiplication from AB explants were observed. Explants showed normal development with no visual indication of phytotoxicity, such as browning or blackening, and they multiplied profusely with no degeneration of tissues or death of explants. Multiplied shoots rooted promptly when transferred to rooting medium, and no morphological abnormalities were observed, such as irregular leaf shape and color or abnormal root development and color.

Induction was observed 15–30 d from culture initiation in most treatments for AB explants, with frequency varying from 4% to 40% (Table 1). Shoot organogenesis was observed over a period of 150 d and varied from 46 to 160 shoots per explant (Table 1). Once initiation was observed, shoots started multiplying, forming clumps with multiple shoots (Fig. 1B). Shoot multiplication was

Table 1. Percentage of shoot induction (after 30 d) and number of shoots per explant (after 150 d) from immature apical flower buds of *Doritaenopsis* with combinations of NAA and TDZ.

Treatment (μM)		Shoot induction (%) ^a	No. of shoots per explant
NAA	TDZ		
0	0	0	0
0	2.3	6.9 d ^b	67.5 c
0	4.5	4.0 d	0
0	9.1	31.0 b	118.5 b
5.4	0	4.0 d	0
5.4	2.3	21.4 c	62.6 c
5.4	4.5	40.0 a	160.0 a
5.4	9.1	30.0 b	155.3 a
10.7	0	4.6 d	0
10.7	2.3	22.6 c	46.3 c
10.7	4.5	30.8 b	151.3 a
10.7	9.1	26.5 c	110.7 b

^aData for percent shoot induction were normalized using arcsin transformation.

^bMean separation within columns by Duncan's multiple range test at $P \leq 0.05$. Means followed by the same letter are not significantly different.

observed 60–90 d from culture induction and was monitored weekly with a final evaluation at 150 d. There were significant differences for the treatments performed whereby both NAA and TDZ used alone or combined (NAA + TDZ) affected the frequency of shoot induction and the number of shoots produced per explant. The treatment lacking plant growth regulators showed no induction response, and therefore no fresh weight was registered. For all treatments producing shoots, no significant differences were observed in mean fresh weight (9.3–15.6 g) after 150 d in culture (data not shown). The highest treatments ranged from 30% to 40% shoot induction and exhibited the highest number of shoots after 150 d (Table 1). The highest shoot induction (40%) was achieved using 5.4 μM NAA + 4.5 μM TDZ (Table 1) and differed significantly from other treatments. The use of TDZ alone at 9.1 μM and the combinations of 10.7 μM NAA + 4.5 μM TDZ and 5.4 μM NAA + 9.1 μM TDZ provided 30%–31% shoot induction and did not differ significantly from each other (Table 1). However, for the number of shoots produced per explant, the combinations of 10.7 μM NAA + 4.5 μM TDZ and 5.4 μM NAA + 9.1 μM TDZ returned higher numbers of shoots per explant than those produced when using TDZ alone at 9.1 μM (Table 1).

In our study, although the best percentage of shoot induction was only 40%, the number of shoots obtained per explant was higher for all treatments (Table 1) when compared with PLB formation. Studies with PLBs have shown similar or higher rates of induction, but with lower regeneration rates. Park et al. (2002) used thin leaf sections of *Doritaenopsis* hybrids [*Doritaenopsis* New Candy $\times$ *Doritaenopsis* (Mary Anes $\times$ Ever Spring)] to induce PLBs. The highest percentage of PLB formation (72.3%) resulted in 18 PLBs per explant. In subsequent studies, Park et al. (2003) achieved 47.2% PLB formation from *Doritaenopsis* hybrid root tips used as explants, but some PLB formation was callus-mediated and only 2–6 PLBs per explant

were observed. Shoot formation ranged from 46 to 160 shoots per explant (Table 1).

In the absence of TDZ, induction percentages were the lowest and no subsequent shoot multiplication was achieved (Table 1). Similarly, Ernst (1994) observed a lack of proliferation in the absence of TDZ for protocorms of *Phalaenopsis* Dora and *Doritaenopsis* George Moler induced from flower stem nodes. In that study, TDZ at 11.4 μM was the best concentration, producing an average of 10 shoots per node for both hybrids. However, no auxin was used. Using *Doritaenopsis* hybrids, Park et al. (2002, 2003) produced PLBs from thin leaf sections and root tip explants using 9.1 and 2.3 μM TDZ alone, respectively. In our study, immature apical flower buds produced the largest number of shoots at concentrations of 9.1 μM TDZ alone, while reduced numbers of shoots were observed with 2.3 μM TDZ alone (Table 1). In contrast, when an auxin (NAA) was used alone, no shoot formation was observed (Table 1).

Chen and Chang (2004) observed that in the presence of 4.5 and 13.6 μM TDZ, NAA significantly decreased somatic embryo formation from seed-derived protocorms of *Phalaenopsis amabilis* var. *Formosa* Shimadzu. However, in our study NAA at 5.4 or 10.7 μM significantly increased the number of shoots formed per explant when in the presence of 4.5 or 9.2 μM of TDZ. TDZ has been demonstrated to successfully induce shoot organogenesis in different orchid species (Chen and Chang, 2001; Nayak et al., 1997). In this study, the effectiveness of TDZ to induce shoot organogenesis from immature apical flower buds of *Doritaenopsis* Purple Gem 'Ching Hua' was addressed through the high number of shoots obtained when TDZ was used alone at 9.1 μM . Furthermore, when TDZ was combined with NAA, an efficient means to induce multiple shoots in vitro was observed, as indicated by the higher number of shoots developed per explant as compared with studies examining PLB formation.

Rooting and plantlet regeneration. Following shoot multiplication, shoots were isolated and transferred to a rooting medium, where 100% of in vitro-induced shoots rooted and regenerated plantlets (data not shown). In some cases, roots formed in the multiplication medium, facilitating the rooting process and producing healthy plantlets. Despite the presence of phenolics in culture, no abnormalities or detrimental effects were observed and shoots developed healthy roots, thus regenerating into plantlets with normal vegetative and reproductive morphology. General developmental stages from unrooted to rooted shoots are shown in Fig. 1C. Fully developed shoots containing well-developed roots (Fig. 1D) were transferred to 10-cm pots containing potting mix and acclimatized under greenhouse conditions. Plantlets were grown to maturity within 8–10 months and showed normal peloric flower morphology (Fig. 1E). No floral off-types were observed. Regenerated plantlets showed 100% survival and establishment in greenhouse.

We have demonstrated an efficient means for in vitro clonal propagation and plant regeneration from immature apical flower buds of *Doritaenopsis* Purple Gem 'Ching Hua' using direct in vitro shoot organogenesis. The high rates of shoot multiplication obtained are essential for the large-scale commercial production of orchids and offer a means for mass clonal propagation of this and possibly other related *Doritaenopsis* hybrids.

Literature Cited

- Arditti, J. and R. Ernst. 1993. Micropropagation of orchids. Wiley, New York.
- Chen, J.T. and W.C. Chang. 2001. Effects of auxins and cytokinins on direct somatic embryogenesis from leaf explants of *Oncidium* 'Gower Ramsey'. *Plant Growth Regulat.* 34:229–232.
- Chen, J.T. and W.C. Chang. 2004. Induction of repetitive embryogenesis from seed-derived protocorms of *Phalaenopsis amabilis* var. *Formosa* Shimadzu. *In Vitro Cell. Dev. Biol. Plant* 40:290–293.
- Ernst, R. 1994. Effects of thidiazuron on in vitro propagation of *Phalaenopsis* and *Doritaenopsis* (Orchidaceae). *Plant Cell Tissue Org. Cult.* 39:273–275.
- Flamee, M. and G. Boesman. 1977. Clonal multiplication of *Phalaenopsis* hybrids by means of sections of flower stalks. *Med. Fac. Landbouw. Rijksunivers. Ghent* 42:1865–1868.
- Gamborg, O.L., R.A. Miller, and K. Ojima. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50:151–158.
- Lin, C.C. 1986. In vitro culture of flower stalk internodes of *Phalaenopsis* and *Doritaenopsis*. *Lindleyana* 1(3):158–163.
- Martin, K.P. and J. Madassery. 2006. Rapid in vitro propagation of *Dendrobium* hybrids through direct shoot formation from foliar explants, and protocorm-like bodies. *Sci. Hort.* 108:95–99.
- Morel, G. 1974. Clonal multiplication of orchids. In: C.L. Withner (ed.). *The orchids: scientific studies*. Wiley, New York.
- Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15:473–497.
- Nayak, N.R., S. Patnaik, and R.P. Rath. 1997. Direct shoot regeneration from foliar explants of epiphytic orchid *Acampae praemorsa* (Roxb.) Blatter and McCann. *Plant Cell Rep.* 16:583–586.
- Park, S.Y., E.C. Yeung, D. Chakrabarty, and K.Y. Paek. 2002. An efficient direct induction of protocorm-like bodies from leaf subepidermal cells of *Doritaenopsis* hybrid using thin-section culture. *Plant Cell Rep.* 21:46–51.
- Park, S.Y., H.N. Murthy, and K.Y. Paek. 2003. Protocorm-like body induction and subsequent plant regeneration from root tips cultures of *Doritaenopsis*. *Plant Sci.* 164:919–923.
- SAS Institute. 1989. SAS/STAT user's guide, version 6. 4th ed. SAS Institute, Cary, NC.
- Tisserat, B. and D. Jones. 1999. Clonal propagation of orchids. *Plant Cell Cult. Protocols* 11:127–134.
- Tokuha, K. and M. Mii. 1993. Micropropagation of *Phalaenopsis* and *Doritaenopsis* by culturing shoot tips of flower stalk buds. *Plant Cell Rep.* 13:7–11.
- Tsukazaki, H., M. Mii, K. Tokuha, and K. Ishikawa. 2000. Cryopreservation of *Doritaenopsis* suspension culture by vitrification. *Plant Cell Rep.* 19:1160–1164.
- USDA. 2006. Floriculture crops—2005 summary. Natl. Agr. Stat. Serv., U.S. Dept. of Agriculture, Washington, DC.