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Increased Ethylene and Decreased Phenolic Compounds Stimulate Somatic Embryo Regeneration in Leaf Thin Section Cultures of *Doritaenopsis* Hybrid

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The effects of endogenous levels of ethylene and phenolic compounds on somatic embryogenesis, medium-browning, and peroxidase activity were evaluated in thin section cultures of *Doritaenopsis*. Cultures were maintained for 8 weeks with four different treatments: i) thick leaf segment culture, ii) thin leaf section culture, iii) thin leaf section culture with ventilation, or iv) thin leaf section culture after explants were first washed. Explants cultured in closed vessels produced a larger number of somatic embryos than those reared in the ventilated vessels. This enhanced formation confirmed the greater involvement of accumulated ethylene under non-ventilated conditions, because wound-induced tissues from thin leaf sections normally release high level of ethylene. When explants were washed in the liquid medium and inoculated on the same solid medium, somatic embryo production was 1.7 and 18.5 times higher than in the thin section cultures and thick segment cultures, respectively. Reducing the level of phenolics in explants at the initial stage of culturing apparently stimulated this embryo regeneration.

Keywords: *Doritaenopsis*, ethylene, peroxidase activity, phenolic compounds, somatic embryo, thin section culture

Genus *Phalaenopsis*, which includes *Doritaenopsis* (*Doritis* x *Phalaenopsis*), has high market value as a source of cut flowers and potted plants throughout the world. Many researchers have developed *in vitro* protocols for their propagation (Arditti and Ernst, 1993). Since the leaf segment culture method was first developed by Tanaka and Sakanish (1977), numerous attempts have been made to regenerate *Phalaenopsis* cultivars and hybrids via somatic embryogenesis (generally termed 'protocorm-like bodies' in Orchidaceae) (Tanaka, 1992; Park et al., 1996, 2000, 2002a, b, 2003; Chen and Chang, 2006). Recently, a thin cell layer approach has been shown to be an efficient regeneration protocol *in vitro*. High-frequency shoot regeneration has been obtained using this system in *Rhynchostylis gigantea* (Le et al., 1999) and *Aranda* Deborah (Lakshmanan et al., 1995).

These culturing techniques generally result in the wounding of plant tissues, which in turn induces the production of ethylene (Abeles et al., 1992; Park et al., 2002b). This response affects phenylpropanoid metabolism via 1-aminocyclopropane-1-carboxylic acid (ACC) (Song et al., 2001; Kim et al., 2004; Wu et al., 2006) and, hence, the accumulation of phenolic compounds (Tang and Newton, 2004). The accumulation of ethylene in a culturing system has been associated with either the inhibition or stimulation of shoot organogenesis and somatic embryogenesis in several plant species (Purnhauser et al., 1987; Cho and Kasha, 1989; Adkins et al., 1990; Chi et al., 1991; Tadeo et al., 1995; Goh et al., 1997; Mundhara and Rashid, 2006). Moreover, phenolic compounds (e.g., chlorogenic acid, dicaffeoyl tartaric acid, and isochlorogenic acid) have been implicated in organ formation from *in vitro* cultures (Arezki et al., 2001; Fernández-Lorenzo et al., 2005).

Peroxidase (E.C.1.11.1.7), a ubiquitous enzyme, plays a significant role in many physiological processes. Its activity increases in response to a variety of physical, chemical, and biological stresses (Faivre-Rampant et al., 1998; Kim et al., 2000; Moon et al., 2005; Wi et al., 2006). This enzyme is also involved in the H_2O_2 detoxification mechanism, in lignification, and in general metabolism of plant phenolics (Lavid et al., 2006).

Previously we reported an efficient and rapid system for somatic embryo regeneration from *Doritaenopsis*, using leaf thin sections obtained from plant-derived flower stalks (Park et al., 2002b). In a continuation of that earlier work, here we have estimated changes in the contents of ethylene and phenolic compounds, as well as in peroxidase activity.

MATERIALS AND METHODS

Plant Material

Doritaenopsis 'New Candy'×*Dtps.* ('Mary Anes'×'Ever Spring') (*Dtps.* hybrid) was used as the culturing source material. Flower stalks bearing the 2nd to 4th nodes from the base, with lateral buds, served as explants. Five-cm-long stalk sections were decontaminated for 20 min in a 3.0% (v/v) sodium hypochlorite solution and washed three times in sterilized distilled water. They were then cultured in 25 × 250 mm tubes containing 20 mL of an MS medium (Murashige and Skoog, 1962) supplemented with 20.2 μM N^6-benzyladenine (BA) and 45 g L^{-1}

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sucrose. The medium was solidified with 7.0 g L^{-1} agar (Duchefa, The Netherlands), and the pH was adjusted to 5.5 before autoclaving for 20 min at 120°C (1.37×10^5 Pa). After 4 weeks of culturing, plantlets that had developed from the lateral bud region were harvested for use as experimental explants.

Thin Section Leaf Cultures

To prepare the thin sections, five to seven 1-mm segments were cut transversely from the proximal (basal) portion of the youngest leaf on each plantlet, as described by Park et al. (2002b). These sections were placed cut-side down on plastic Petri dishes (10-cm diam.) that contained a 1/2 MS solid medium supplemented with 9.0 μM thidiazuron, 20% (v/v) coconut water, and 10 mg L^{-1} adenine sulfate, and which was solidified with 2.3 g L^{-1} gelrite. These experiments were designed to examine the role of ethylene and phenolics on somatic embryo induction from leaves of the *Dtps.* hybrid. For monitoring ethylene under Treatment FS, 10-mm-diameter holes were made in the lid of a culture vessel (10-cm Petri dish) that was then covered with a 0.22 μm millipore filter for ventilating the internal gases, mainly ethylene. To investigate the effect of phenolic compounds under Treatment WS, leaf thin sections were washed for 2 h with a liquid medium, then inoculated on the same kind of solid medium in non-ventilated dishes. As a control, thick (5 × 10 mm) leaf segments (Treatment C) and thin leaf sections (CS) were placed on the same type of media, in non-ventilated dishes. All cultures were incubated at 25 ± 1°C, under a 16-h photoperiod from cool-white fluorescent lighting (Kumho FL40D, Korea) of 30 μmol m^{-2} s^{-1} photosynthetic photon flux. After 8 weeks of culturing, the explants were evaluated for the percentage of somatic embryo formation, and numbers of somatic embryos, callus-like nodules, and direct shoots per explant. To analyze the biochemical changes that occurred during this regeneration stage, samples were collected at week 0, 4, and 8 after culturing.

Ethylene Measurement

For our ethylene measurements, 0.3 mL of air was extracted from the vessel via the hole in the lid. The analysis was performed with a gas chromatograph (HP 6890; Hewlett Packard, USA) equipped with a poropack-Q column, a flame ionization detector, and an integrator. Operational temperatures for the oven, injector, and detector were 100, 150, and 200°C, respectively (Goh et al., 1997). N_2, at a flow rate of 30 mL min^{-1}, was used as the carrier gas.

Phenolic Compounds in Explants and Medium-Browning

To determine the content of total phenolics in the explant, 100 mg of fresh tissue was collected weekly and stored at -70°C. These frozen samples were then homogenized with 50 μL (80%) ethyl alcohol (extract solution), followed by the addition of 1 mL folin-ciocalteu phenol reagent and 5 mL sodium carbonate (10%). After 20 min, the absorption of the mixture was read at 735 nm with a spectrophotometer (Uvikon 930; Kontron, Switzerland). The absorbance value represented the phenolic content, based on a standard curve of 0, 20, 40, 60, and 80 mg L^{-1} garlic acid. Medium-browning was also assessed at the end of the culture period by homogenizing 5-g samples with 12.5 mL (80%) ethyl alcohol, followed by shaking for 24 h at 100 rpm to extract the oxidative phenolics. The absorption of the mixture was read spectrophotometrically at 420 nm.

Peroxidase Activity

To measure peroxidase activity, 100 mg of fresh tissue was collected weekly and stored at -70°C for analysis (Kim et al., 2000). The samples were ground with liquid nitrogen and 0.5 mL Tris buffer (50 mM, pH 7.5) that had been supplemented with 1% (w/v) insoluble polyvinyl-pyrrolidone (PVP). They were then centrifuged for 15 min at 11000*g* (4°C). Afterward, 50 μL of the supernatant was reacted with 2.95 mL potassium phosphate buffer (0.1 M, pH 6.2). Absorbance of the mixture was read spectrophotometrically at 420 nm (20 s delay time and 1 min measure time).

Experimental Design and Data Analysis

Experiments were carried out in a randomized design and each treatment was repeated twice with five replicates. Data were subjected to Duncan's multiple range test and SAS analysis (SAS Institute, USA). Mean values were considered significantly different at $P \leq 0.05$.

RESULTS AND DISCUSSION

To determine how ethylene and phenolic compounds might influence the production of somatic embryos, callus-like nodules, and shoots, we applied four types of treatment to *Doritaenopsis* cultures over 8 weeks (Table 1). When the leaf explants were first washed in a liquid medium and then inoculated on the same solid medium in a non-ventilated vessel (WS), somatic embryo production was greatest, being 1.7 times higher than for the thin section cultures (CS) and 18.5 times higher than for conventional thick segment cul-

Table 1. Effect of culture method on somatic embryo induction from thick and thin sections of *Dtps.* hybrid leaves after 8 weeks of culture.

Treatment[z]	Number of regenerants per explant		
	Somatic embryos	Callus-like nodules	Shoots
Thick segment culture (C)	0.87 d[y]	0.53 d	0.59 b
Thin section culture (CS)	9.67 b	14.73 a	1.27 a
Thin section culture with ventilation (FS)	5.27 c	8.47 b	0.53 b
Thin section culture after washing (WS)	16.07 a	4.53 c	1.40 a

[z]For C, CS, and WS treatments, explants were cultured in vessels without holes; for FS treatment only, in ventilated vessel without hole.
[y]Mean separation within columns by Duncan's multiple range test ($P \leq 0.05$).

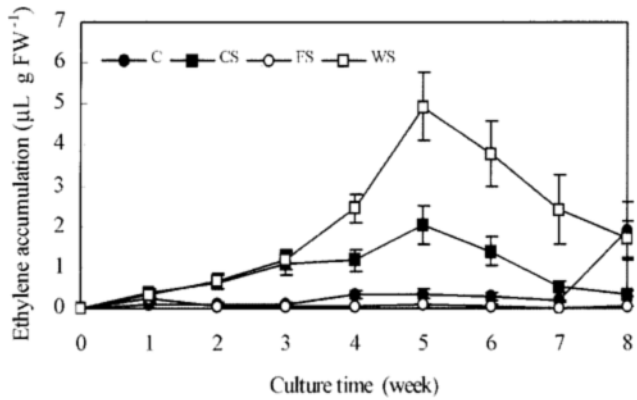

Figure 1. Changes in ethylene contents within culture vessels over 8 weeks of various treatments with *Dtps.* hybrid. (C, thick segment culture; CS, thin section culture; FS, thin section culture in ventilated vessel; WS, thin section culture after washing of explant). Bars represent ± SE.

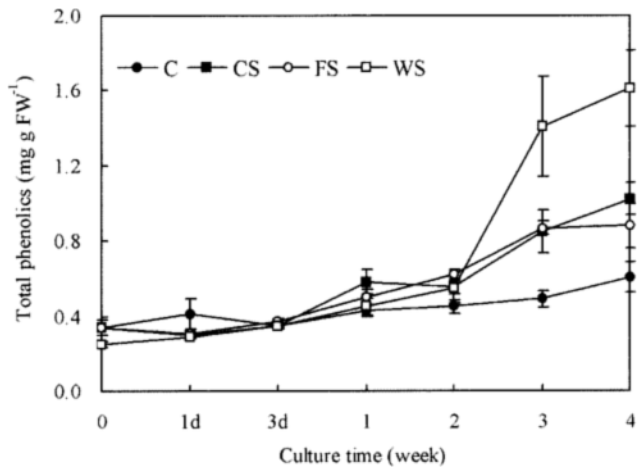

Figure 2. Changes in total phenolics in explants during 8 weeks of culture (C, thick segment culture; CS, thin section culture; FS, thin section culture in ventilated vessel; WS, thin section culture after washing of explant). Bars represent ± SE.

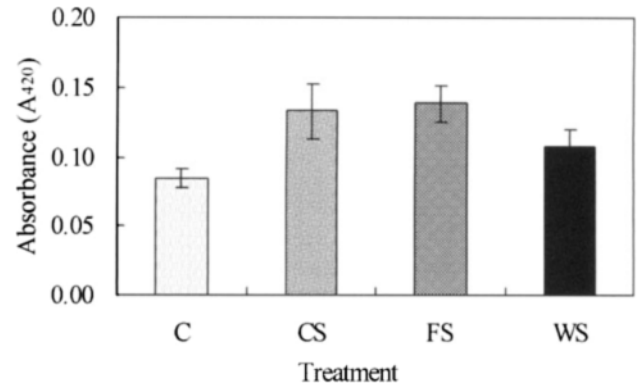

Figure 3. Medium-browning by phenolics in each treatment after 8 weeks of culture (C, thick segment culture; CS, thin section culture; FS, thin section culture in ventilated vessel; WS, thin section culture after washing of explant). Bars represent ± SE.

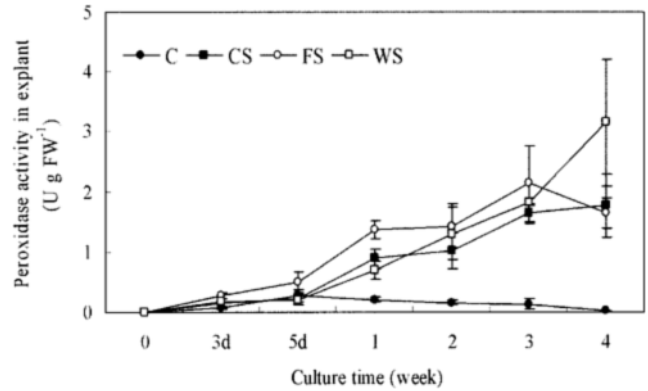

Figure 4. Changes in peroxidase activity during culture period (C, thick segment culture; CS, thin section culture; FS, thin section culture in ventilated vessel; WS, thin section culture after washing of explant). Bars represent ± SE.

tures (C). Explants cultured in ventilated vessels did not produce any more embryos than did those in closed vessels. Some regenerants eventually developed into callus-like nodules or became necrotic. In the WS treatment, ethylene production began to increase from 1 week after culturing, remarkably increasing in the 3rd week and peaking at Week 5. Changes in vessel ethylene concentrations over that period were closely correlated with the percentage of somatic embryos that formed explants (Fig. 1). This indicates that somatic embryo formation was stimulated by ethylene being released from thin leaf sections while inhibited when ethylene was removed through ventilation. Moreover, the latter scenario resulted in 1.7 times greater callus-like nodule formation than was found in the non-ventilated vessels.

The role of ethylene in plantlet regeneration *in vitro* tends to depend on the particular species. For example, this hormone stimulates shoot morphogenesis in rice calli (Adkins et al., 1990) and embryogenesis from anther cultures of barley (Cho and Kasha, 1989). In contrast, ethylene appears to be inhibitory to *in vitro* shoot regeneration in *Triticum* (Purnhauser et al., 1987), mangosteen (Goh et al., 1997), *Citrus* (Tadeo et al., 1995), and *Brassica* (Chi et al., 1991) as well as to somatic embryogenesis in carrot (Roustan et al., 1989). Our experimental observations showed that more ethylene was detected from thin section cultures than from the thick leaf segment cultures. A good correlation between changes in ethylene content and the time of somatic embryo regeneration also indicates a positive signal for embryo induction during thin section culturing.

In the initial stage (before inoculation), phenolic compounds were more abundant in non-washed explants (0.23 mg g^{-1} FW) than in those washed with the liquid medium (0.17 mg g^{-1} FW). However, after 1 d in culture, the phenolic content in thin-sectioned, unwashed explants decreased to a level less than that measured from the washed tissues (Fig. 2). We can postulate that the phenolic compounds that had been released immediately to the medium from the cut side of the explant had then been oxidized, causing the browning of the medium (Fig. 3). At Week 4, the level of phenolics was exactly correlated with the number of somatic embryos produced, which directly demonstrates that both peroxidase activity and metabolism are related to regeneration potential. Ernst (1994) has reported that, when explants of *Phalaenopsis* and *Doritaenopsis* are cultured *in vitro*, frequent transplanting to fresh media is desirable because these genera release substantial amounts of phenolics, which then oxidize and become phytotoxic. In view of the

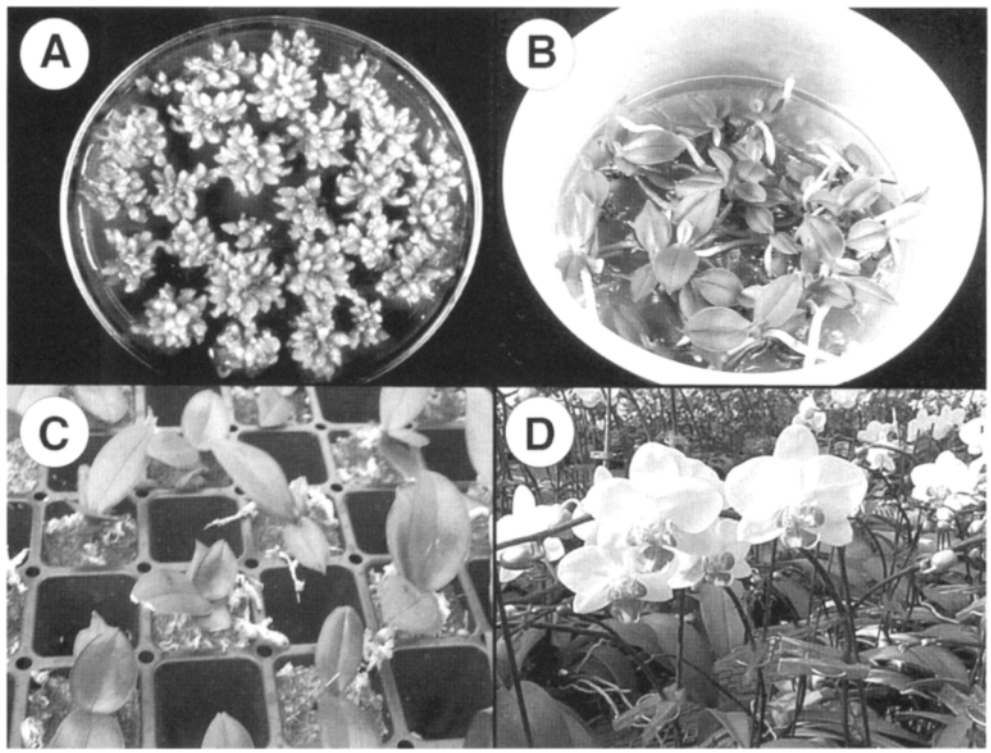

Figure 5. Clonal proliferation via somatic embryogenesis of *Dtps.* hybrid from floral stalk-derived leaves. **A.** Mass of somatic embryos (Protocorm-like bodies; PLBs) developing from leaf thin section culture. **B.** Plantlets growing on modified Hyponex medium (6.5N-4.5P-19K 1 g L^{-1} + 20N-20P-20K 1 g L^{-1} + peptone 2 g L^{-1} + 3% (w/v) potato homogenate + 0.5% activated charcoal + 30 g L^{-1} sucrose). **C.** Acclimatized plants in greenhouse. **D.** Flowering after 12 months of acclimatization.

results so far achieved, it is possible that reducing the amount of phenolics from explants in the initial stage of culturing will stimulate somatic embryo regeneration. Here, after two weeks of culturing, all treatments, but especially WS, showed remarkably higher phenolic contents. By that time (Weeks 2 to 3), the cut surfaces of the explants had cured and new regenerants were being formed. In plants, total phenolics also play a role as a lignin precursor via polymerization, under natural lignification processes (Strack et al., 1989).

Our thin leaf sections (Treatments CS, FS, and WS) showed higher peroxidase activities throughout the regeneration period, beginning after Day 5 (Fig. 4). Activity was highest in FS cultures for the first 3 weeks, but then declined at Week 4, while those from the WS treatment showed continuously increasing levels that peaked at Week 4 before dropping later in the culturing period.

Peroxidases have been implicated in phenolic compound oxidation in a wide range of cellular reactions. Our results also confirmed this, with similar tendencies being plotted in the curves for peroxidase and phenolics, especially from the FS treatment. This greater peroxidase activity from *in vitro* plants is due to higher activities of both acidic (anionic) and basic (cationic) isoperoxidases (Faivre-Rampant et al., 1998). The former is involved in lignification while the latter is usually associated with auxinic catabolism. Faivre-Rampant et al. (1998) have also reported that higher peroxidase activity and incidences of lignification and ethylene production are involved in growth reductions. Lamport (1986) has shown that peroxidases tend not to occur in regions with the highest growth and that their activities are indeed lowest in the growing apical portion of the shoot. Therefore, we might infer that peroxidase activity in already-regenerated plants will be low if they are grown under proper, stress-free conditions. Finally, Lee and Lee (2000) have found that peroxidase specific activity is higher in the calli than in the mature leaves in *Geranium*.

Peroxidases can reinforce the cell wall through the deposition of several cell wall components, such as lignin, suberin, and extensin (Imaseki, 1985). During the regeneration stage (i.e., active cell proliferation), peroxidase activity can be higher than at the growth stage (after regeneration is completed) because during the former, physiological metabolism of the explant becomes active. Our WS treatment, which produced numerous somatic embryos, showed the highest peroxidase activity, and the available data indicated a positive relationship between increased ethylene production and a greater content of phenolic compounds and peroxidase activity. This response to explant wounding, i.e., more peroxidase activity and polyphenol accumulations, subsequently prompted ethylene production, thereby triggering somatic embryo regeneration.

In the final phase of these experiments, the plantlets that had developed from somatic embryos were potted into sphagnum moss and acclimatized in the greenhouse (Fig. 5A, B and C) where they grew well and blossomed 12 months after their transplantation (Fig. 5D).

We can now conclude that: 1) ethylene stimulates the formation of somatic embryos; 2) the removal of endogenous phenolic compounds in the leaves, via washing, remarkably

increases the frequency of this formation and decreases the incidence of medium-browning; and 3) peroxidases are implicated in the oxidation of phenolic compounds.

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