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DOES ETHYLENE PLAY A ROLE IN THIDIAZURON-REGULATED SOMATIC EMBRYOGENESIS OF GERANIUM (*PELARGONIUM* × *HORTORUM* BAILEY) HYPOCOTYL CULTURES?

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SUMMARY

The accumulation of ethylene in headspace of hypocotyl cultures of geranium (*Pelargonium* × *hortorum* Bailey) and its possible role in thidiazuron-mediated somatic embryogenesis was investigated. The action of ethylene as determined by various ethylene synthesis and action inhibitors was varied. Silver nitrate ($AgNo_3$), aminoethoxyvinylglycine (AVG), and silver thiosulphate (STS) had no significant influence on the embryogenic response, while 1-methylcyclopropene (1-MCP) applied during the initial 3 d of induction or the expression phase, significantly increased the number of somatic embryos formed. Thidiazuron-treated tissues accumulated large quantities of ethylene within 6 h of culture, but the levels decreased after 12 h and reached very low levels after 3 d in culture. In the presence of acetylsalicylic acid (ASA), the levels of ethylene decreased by 20 to 50% during the first 48 h of culture. Analysis of endogenous auxin, cytokinins, and abscisic acid (ABA) indicated possible interactions of ethylene with other phytohormones during the induction of somatic embryos on geranium hypocotyl explants. Thidiazuron (10 μ*M*) increased, while ASA decreased the levels of endogenous auxin, cytokinins, and abscisic acid during this period of induction.

Key words: ethylene inhibitors; geranium; somatic embryogenesis; aspirin (acetylsalicylic acid); endogenous plant growth regulators.

INTRODUCTION

Thidiazuron (N'phenyl-N'-1,2,3-thidiazol-5-ylurea; TDZ), a phenyl urea derivative, has been shown to provide sufficient stimulus for the induction of somatic embryogenesis in a variety of plant species including peanut, tobacco, and geranium, substituting for auxin or combined auxin and cytokinin requirements of embryogenesis (Gill and Saxena, 1992, 1993; Visser et al., 1992). One of the mechanisms for the observed promotion of somatic embryo production by TDZ may be through elevated levels of endogenous auxins, cytokinins, and abscisic acid (ABA) observed in peanut seed (Murthy et al., 1995) and geranium (*Pelargonium* × *hortorum* Bailey) hypocotyl tissues (Hutchinson et al., 1996a, 1996b; Hutchinson and Saxena, 1996b). However, we have previously reported an increase in the frequency and synchronization of TDZ-regulated geranium somatic embryo production by acetylsalicylic acid (ASA; aspirin) (Hutchinson and Saxena, 1996a), a compound that may act as an ethylene synthesis inhibitor (Leslie and Romani, 1986). These observations indicate that ethylene may play an important role during TDZ-mediated somatic embryogenesis. We hypothesize that TDZ may have enhanced ethylene production leading to elevated levels, which in turn was inhibitory to somatic embryogenesis, and the addition of ASA may have released the induced embryos from this ethylene-stimulated inhibition. It has been shown that TDZ-induced leaf abscission in cotton was mediated, at least in part, by an increase in endogenous ethylene production (Suttle, 1984). Ethylene was also found to increase in mung bean hypocotyl suspension cultures treated with TDZ (Yip and Yang, 1986). However, the role of ethylene in somatic embryogenesis has not been clearly understood.

Ethylene has been reported to have both promotive and inhibitory roles on embryogenesis depending on the species in question (Biddington, 1992). Embryo induction or further embryo development in carrot callus and orchard grass leaf cultures has been reported to be inhibited by ethylene (Songstad et al., 1989). As well, ethylene-synthesis or ethylene action inhibitors including silver nitrate, $AgNO_3$ have been used to increase the rate of somatic embryogenesis by reducing ethylene production or ethylene action (Beyer, 1976; Biddington et al., 1988; Vain et al., 1989a, 1989b; Roustan et al., 1990; Songstad et al., 1991) and aminoethoxyvinylglycine (AVG) (Robinson and Adams, 1987; Roustan et al., 1990). Similarly, salicylic acid (SA) and ASA have been shown to inhibit ethylene production and promote embryo formation in carrot cell cultures (Roustan et al., 1990). In contrast, low concentrations of ethephon (0.01–1.0 mg/l) increased embryo production in citrus ovular callus cultures, although higher concentrations proved inhibitory (Kochba et al., 1978). Although ethylene may have positive effects on somatic embryogenesis, recent experiments involving transgenic plants illustrate the complex interactions between this phytohormone and other hormones that cannot be separated easily into its component parts. The synthesis and activities of auxin, cytokinin, and ethylene, for example, have been shown to be closely interrelated (Klee and Romano, 1994).

The objectives of the present study were to investigate the possible role of ethylene in TDZ-mediated somatic embryo induction and de-

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velopment in geranium hypocotyl cultures and to elucidate the possible changes of ethylene and other phytohormones such as auxin, cytokinin, and ABA. In the presence of TDZ, ethylene and other phytohormones accumulate, while in the presence of ASA, these phytohormones decreased in hypocotyl cultures of geranium. The results of the present study indicate possible complex interactions between ethylene and the other phytohormones; auxin; cytokinin, and ABA, during somatic embryo induction.

Materials and Methods

Seed Cultures

Seeds of the diploid Zonal geranium, *Pelargonium* × *hortorum* Bailey cv. Scarlet Orbit Improved (Stokes Seed Co., Catherines, ON), were surface sterilized by immersing in 95% ethanol for 45 s and then immersing in 1.5% sodium hypochlorite solution containing Tween 20 (2 drops/100 ml solution). Seeds were agitated continuously in the sodium hypochlorite solution for 20 min and then rinsed three times with sterile deionized water. Fifteen seeds were germinated aseptically in each 100 × 15 mm disposable petri dish containing 25 ml water-agar (0.85% purified agar, Sigma [Sigma Chemical Co., St. Louis, MO] in distilled water). The petri dishes were sealed with parafilm and incubated in the dark at 25° C for 6 d.

Hypocotyl Cultures

Six-d-old etiolated hypocotyls were cut into four or five 8-mm long segments. Ten explants were cultured per 100 × 15 mm disposable petri dish containing 25 ml of media. The culture medium consisted of MS (Murashige and Skoog, 1962) salts, B5 (Gamborg et al., 1968) vitamins, and 30 g l^{-1} sucrose. The explants were placed on the induction medium consisting of 10 μ*M* TDZ (induction medium; IM) with or without various concentrations of ethylene inhibitors; ASA, 10 μ*M*; AVG, 1, 5, 10, 20 μ*M*; silver nitrate ($AgNO_3$), 2.5, 5, 10, 20 μ*M*; silver thiosulphate (STS), 0.2, 1, 5 μ*M*; 1-methylcyclopropene (1-MCP), 1.2, 12, and 60 ppm. Silver thiosulphate stock solution was prepared with a molar ratio of 1:4 between silver and thiosulphate. A 0.02 *M* STS stock solution was prepared by slowly adding 20 ml of 0.1 *M* $AgNO_3$ stock solution into 80 ml of 0.1 *M* sodium thiosulphate stock solution. For the addition of 1-MCP, a tiny hole was bored in the petri dish cover and sealed with an airtight rubber septum that automatically resealed after addition of the compound. An appropriate amount of 1-MCP, which resulted in the final desired concentration, was injected into the culture headspace using a sterile syringe. All cultures were maintained for 3 d on the induction medium and then transferred to basal medium (expression medium; EM).

To evaluate the effect of the ethylene inhibitors on growth and development of the induced embryos, hypocotyl explants were maintained for 3 d on IM and subsequently on EM supplemented with similar concentrations of AVG, $AgNO_3$, STS, or 1-MCP as above. All the supplements were added after autoclaving the media. The pH of the media was adjusted to 5.5 ± 0.1 before autoclaving at 1.10 kg cm^{-2} for 20 min.

To investigate the effect of pH on somatic embryo development, the pH of the medium was adjusted to 3, 4, 5, 6, or 7 before autoclaving. The fluctuations of pH after autoclaving were less than 10%, and so the pH was not adjusted after autoclaving. The cultures were placed in a growth chamber set at 25° C and illuminated (16-h photoperiod; 70–78 μmol m^{-2} s^{-1}) by cool-white fluorescent tubes. The mean number of somatic embryos per hypocotyl section were determined after 16 and/or 30 d of culture.

Determination of ethylene levels. For the determination of ethylene, 10 hypocotyl segments were placed in 100 × 15 mm disposable petri dishes containing 25 ml of medium consisting of 10 μ*M* TDZ (IM) with or without 10 μ*M* ASA. Petri dishes were sealed with three layers of parafilm. Tiny holes were bored and sealed on the petri dish covers as described earlier. Ethylene concentration in the 50 ml headspace was determined after 6, 12, 24, 48, and 72 h in culture, the period corresponding to the induction period of somatic embryogenesis of geranium (Hutchinson and Saxena, 1996a). A 3 ml gas sample was withdrawn from the airspace above the cultures using a hypodermic syringe and injected into the sampling valve of a gas chromatograph (Hewlett Packard, 5880A series) equipped with a flame ionization detector. The amount of ethylene was calculated from the concentration of gases in the headspace relative to a known standard. Each treatment was replicated three times and the experiments were repeated at least twice.

Endogenous plant growth substance analysis. Analytical validation and methods were similar to those published previously (Murthy et al., 1995). Hypocotyl explants cultured on media supplemented with 10 μ*M* TDZ with or without 10 μ*M* ASA were sampled daily during the first 3 d of culture corresponding to the induction period and stored at −80° C until time for analysis. The samples for the hormone analysis constituted 50 hypocotyl explants harvested from five petri dishes that were randomly selected. Samples were extracted in 4 ml of 80% methanol and agitated on an orbital shaker (Lab-line Instruments, Melrose Park, IL) for 24 h at 4° C in the dark followed by centrifugation (IEC HN-SII centrifuge, International Equipment Co., Needham Heights, MA) for 10 min at 2000 g_n to remove particulate matter. The supernatant was then dried under vacuum using SpeedVac (SpeedVac model DD-20, Precision Scientific, Chicago, IL). The extracts were resuspended in sample diluent (0.5 mmol·l^{-1} phosphate buffer, pH 7.4 with 5% acetonitrile), and an aliquot of 20 μl of each sample was injected into the HPLC system for analysis of auxins, cytokinins, and ABA. The HPLC equipment and elution gradient were the same as described earlier (Murthy et al., 1995). The elution gradient was described to exclude most of other potentially co-chromatographing compounds in the geranium plant extracts. To determine recovery of the plant growth regulators, samples were spiked, several times, with known amounts of standards. Recovery of all the plant growth regulators analyzed was greater than 90%. The percent recovery was obtained by repeated injections of spiked samples over a period of time. In the repeated series of experiments, one tissue sample was split into two groups that were then used to generate recovery data and information. A known amount of standard was added to one replicate of the group samples prior to freeze drying. The sample and the control tissue was compared to the amount of standard added to give the percent recovery.

Identification of the plant growth regulators was verified by gas chromatography-mass spectroscopy (GC-MS) as outlined in Murthy et al. (1995). Briefly, fractions of effluent from the high-performance liquid chromatography (HPLC) system were collected every 30 s by a sample collector and dried using a SpeedVac (SpeedVac model SVC200). The samples were re-suspended in a solution of 0.1 *M* HCl in methanol (Fisher Scientific, Toronto, ON) with 250 μl boron trifluoride. Samples were heated in capped tubes in a heating block at 85° C for 30 min and then cooled to room temperature. This was followed by extraction with 3–5 ml of petroleum ether and evaporation under N_2 with 200 μl hexane until all that remained was hexane (Fisher Scientific).

Samples were analyzed on a Hewlett Packard 5890 Series II Gas Chromatograph including HP 7673 GC/SFC Injector, Edwards 2 Stage Vacuum Pump, HP 7673 Controller, HP 5971 Series Mass Selective Ion Detector, HP MSChem Station (DOS series) software and HP Laserjet III (Hewlett Packard Co., Palo Alto, CA). The column was an HP-5MS Crosslinked 5% Ph Me silicone filament with an integrated diameter of 0.25 mm, length 30 m, film thickness 0.25 μm, and phase ratio 250 (Hewlett Packard Co.). Samples were run over a temperature gradient from 40 to 150° C at 20° C min^{-1} followed by a temperature increase to 250° C at 10° C min^{-1} for a total run time of 20 min. The carrier gas was helium. Ions were detected over the range of 50–350 atomic mass units for the entire run. An internal library for compounds was created by injection of standards, and the ion breakdown products were compared for standards and column fractions to identify the fraction containing the growth regulator.

The growth regulators were quantified by comparison of peak area to regression equations calculated from repeated injections of standards of zeatin, dihydrozeatin (DHZ), indoleacetic acid (IAA), ABA, isopentenyladenine (2iP), tryptamine, adenine, adenosine, (Sigma Chemical Co., St. Louis, MO), and TDZ (NOR-AM Chemical Co., Wilmington, DE).

In all experiments, each treatment consisted of three to four replications and all experiments were repeated at least twice. Data were analyzed using variance (General Linear Model Procedure of PC; SAS Institute Inc., 1995) and the means were compared by least significant differences (LSD) at 5% level of probability.

Results and Discussion

Thidiazuron and ASA significantly influenced the amount of ethylene accumulating in the culture headspace, as well as the endogenous levels of auxin, cytokinin, and ABA. Ethylene levels in the headspace of cultures, basal media (MSO), and TDZ + ASA (TASA) increased during the first 12 h of culture and then decreased to

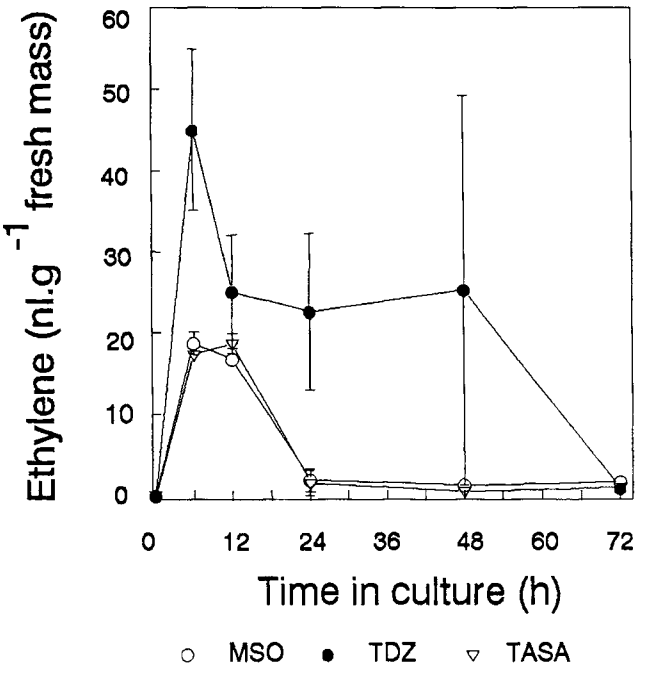

FIG. 1. Effect of thidiazuron (TDZ, 10 μ*M*) alone, or in combination with acetylsalicylic acid (ASA), on the levels of ethylene (nl g^{-1} fresh mass) accumulating in the culture headspace of the hypocotyl cultures of geranium (*Pelargonium* × *hortorum* Bailey). Means were compared by the Least Significant Differences (LSD) test at $P = 0.05$. MSO = basal MS medium; TDZ = MS medium + 10 μ*M* TDZ; TASA = MS medium + 10 μ*M* TDZ + ASA.

negligible levels during the rest of the culture period (Fig. 1). Of significance was the observation that the level of ethylene accumulating in the headspaces of the cultures treated with TDZ was high and remained significantly higher during most of the 3-d induction period compared to those on MSO and on media containing both TDZ and ASA (Fig. 1). The observation of ethylene elevation during the initial 12 h of culture, even in the absence of any plant growth regulators, indicates that the wounding of the explant and the culture conditions could account for part of the ethylene accumulating in the cultures. However, in the presence of TDZ, high levels of ethylene accumulate, even after accounting for the wound-ethylene (Fig. 1). Addition of ASA to the TDZ-containing induction media suppressed the ethylene accumulation to levels similar to MSO controls (Fig. 1). These results indicate that TDZ promotes while ASA inhibits the accumulation of ethylene during somatic embryo induction on geranium hypocotyls.

As a further test of the involvement of ethylene in the action of TDZ, ethylene synthesis and ethylene action inhibitors were added to either the induction media containing TDZ or the expression media lacking TDZ. The addition of ethylene action inhibitors, silver (from silver nitrate and silver thiosulphate) and 1-MCP gave varied results. Silver nitrate (2.5–20 μ*M*) had no significant influence on the number of somatic embryos formed on each explant (range of 12–17 embryos per explant; data not shown), while silver thiosulphate (0.2, 1, 5 μ*M*) significantly decreased the embryogenic response (Table 1) and increased browning of the explants. The STS inhibition was more pronounced when STS was added together with 10 μ*M* TDZ during the

TABLE 1

EFFECT OF VARIOUS CONCENTRATIONS OF SILVER THIOSULPHATE (STS) ON THE MEAN NUMBER OF SOMATIC EMBRYOS FORMED ON HYPOCOTYL EXPLANTS OF GERANIUM (*PELARGONIUM X HORTORUM* BAILEY)[1]

STS concentration (μ*M*)	Day 16		Day 30	
	Induction	Expression	Induction	Expression
0	4.20^{bc}	4.20^{bc}	25.97^{a}	25.97^{a}
0.2	1.80^{cd}	8.30^{a}	8.83^{cd}	22.80^{a}
1.0	0.50^{d}	7.07^{ab}	4.40^{d}	19.80^{ab}
5.0	3.10^{cd}	6.83^{ab}	15.13^{bc}	13.80^{bc}

[1]The explants were maintained on an induction medium supplemented with 10 μ*M* thidiazuron (TDZ) alone or in combination with various concentrations of silver thiosulphate (STS) for 3 d and then transferred to expression medium with or without STS. Means were compared using Least Significant Differences (LSD) test at $P = 0.05$. Means within each column, followed by the same superscript letter, are not significantly different.

TABLE 2

EFFECT OF VARIOUS CONCENTRATIONS OF 1-METHYLCYCLOPROPENE (1-MCP) ON THE MEAN NUMBER OF SOMATIC EMBRYOS FORMED ON HYPOCOTYL EXPLANTS OF GERANIUM (*PELARGONIUM X HORTORUM* BAILEY)[1]

Concentration of 1-MCP (ppm)	Day 16		Day 30	
	Induction	Expression	Induction	Expression
0	5.86^{b}	5.86^{b}	25.97^{b}	25.97^{b}
1.2	10.87^{a}	11.07^{a}	52.43^{a}	41.53^{a}
12	10.63^{a}	9.47^{ab}	37.15^{ab}	42.00^{a}
60	8.05^{ab}	9.37^{ab}	30.73^{b}	36.47^{ab}

[1]The explants were maintained for 3 d on an induction medium with or without various concentrations of the ethylene antagonist, 1-MCP and subsequently transferred to the expression medium with or without similar concentrations of 1-MCP. Means were compared using Least Significant Differences (LSD) test at $P = 0.05$. Means within each column, followed by the same superscript letter, are not significantly different.

3-d period of somatic embryo induction (Table 1). Interestingly, the addition of the ethylene antagonist, 1-MCP (Serek et al., 1994), to either the induction or expression media, increased the number of somatic embryos formed on each explant (Table 2). About twice the number of somatic embryos were formed when 1-MCP was added to the induction medium at a concentration of 1.2 ppm (Table 2). Explants cultured on TDZ and transferred to EM supplemented with various concentrations of 1-MCP formed an average of 37 to 42 somatic embryos, while those transferred to basal expression medium formed an average of 26 somatic embryos after 30 d of culture (Table 2). A similar approach was used to determine whether TDZ action was blocked by AVG, an inhibitor of ethylene biosynthesis via ACC synthase (Yang and Hoffman, 1984). Low concentrations of AVG slightly improved embryogenesis while high concentrations inhibited embryogenesis (Fig. 2). AVG has been found to be very toxic in certain tissues and not to always block ethylene synthesis (Songstad et al., 1989; Biddington, 1992).

A possible pH effect as a mode of action for ASA was ruled out because the cultures maintained on induction medium consisting of TDZ alone or TDZ and ASA had a similar number of somatic embryos

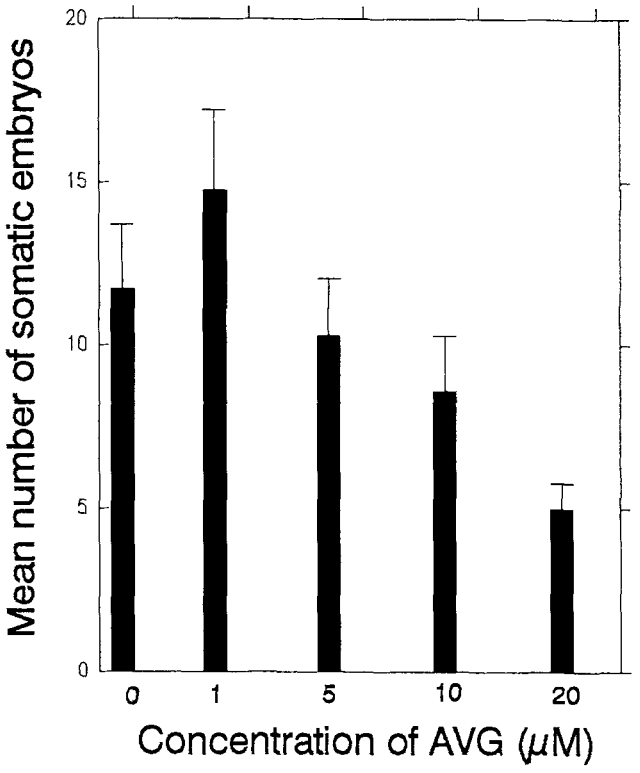

FIG. 2. The mean number of somatic embryos formed on each hypocotyl explant of geranium (*Pelargonium* × *hortorum* Bailey) induced with 10 μ*M* thidiazuron (TDZ) alone or in combination with various concentrations of the ethylene inhibitor, aminoethoxyvinylglycine (AVG). Explants were maintained on the induction medium for 3 d and subsequently transferred to basal medium lacking any growth regulators. Means were compared by the Least Significant Differences (LSD) test at $P = 0.05$.

after 16 d in culture at pH values between 4 to 7 (Table 3). The explants maintained on media with pH adjusted to 3 all browned and died within 16 d in culture. Furthermore, the cultures maintained on basal media with pH adjusted to 3, 4, 5, 6, or 7 failed to form any structures (Table 3). It is likely that the low pH was too acidic to the geranium cultures, while embryogenic response did not appear to be

TABLE 3

EFFECT OF PH ON THE MEAN NUMBER OF SOMATIC EMBRYOS FORMED ON HYPOCOTYL EXPLANTS OF GERANIUM (*PELARGONIUM X HORTORUM* BAILEY).[1]

	MEDIA		
pH	MSO	TDZ	TDZ + ASA (TASA)
3	0[d]	0.47[d]	0.10[d]
4	0[d]	19.10[a]	15.87[a]
5	0[d]	11.53[b]	9.87[bc]
6	0[d]	16.23[a]	16.17[a]
7	0[d]	15.70[a]	6.86[c]

[1]The explants were maintained for 3 d on an induction medium supplemented with 10 μ*M* thidiazuron (TDZ) alone or in combination with 10 μ*M* acetylsalicylic acid (TASA). The pH of the media were adjusted to various pH levels before autoclaving. A variation of 10% in pH values was observed after autoclaving. The explants were transferred to expression medium with similar pH as the induction medium. Means were compared using Least Significant Differences (LSD) test at $P = 0.05$. Means followed by the same superscript letter are not significantly different. MSO = basal MS medium.

adversely affected by pH valued between 4.0 to 7.0 (Table 3). However, at high pH, cultures maintained in media supplemented with TDZ and ASA had a significantly lower number of somatic embryos when compared to culture maintained on medium supplemented with TDZ alone (Table 3). In embryogenic development of carrot cultures, a low pH of 4.0 was found to sustain the multiplication of preglobular stage proembryos (PGSPs), while a pH of 5.0–6.0 provoked PGSPs to develop further (Smith and Krikorian, 1990).

The presence of ASA in the TDZ-supplemented IM reduced the levels of endogenous auxin, cytokinin, and ABA during geranium somatic embryo induction. Thidiazuron alone elevated while ASA decreased the levels of endogenous IAA, tryptamine, zeatin, DHZ, 2iP, and ABA during Day 1 of culture (Table 4). On the second day of culture, the plant growth regulator levels decreased, and by the third day, there were no significant differences in the levels between the cultures maintained on TDZ alone, TDZ with ASA, and the controls (MSO) (Table 4). Acetylsalicylic acid decreased the levels of endogenous auxin, cytokinin, and ABA during this 3-d period of somatic embryo induction (Table 4). How TDZ elevates, while ASA

TABLE 4

EFFECT OF THIDIAZURON (TDZ) WITH OR WITHOUT ACETYLSALICYLIC ACID (ASA) ON ENDOGENOUS PROFILES OF PLANT GROWTH SUBSTANCES (NMOLES.g^{-1} DRY MASS) IN HYPOCOTYL CULTURES OF GERANIUM (*PELARGONIUM X HORTORUM* BAILEY)[1]

		DAY 1			DAY 2			DAY 3		
PGS	Day 0	MSO	TDZ	TASA	MSO	TDZ	TASA	MSO	TDZ	TASA
ADE	2.48	2.04[c]	3.57[a]	2.70[b]	2.19[b]	2.98[a]	2.44[ab]	1.65[a]	1.53[a]	2.12[a]
ADO	1.99	1.63[c]	2.86[a]	2.16[b]	1.75[b]	2.39[a]	1.95[ab]	1.32[a]	1.22[a]	1.70[a]
DHZ	1.75	1.44[c]	2.52[a]	1.92[b]	1.55[b]	2.12[a]	1.72[ab]	1.17[a]	1.08[a]	1.50[a]
ZEA	28.7	23.6[c]	41.2[a]	31.19[b]	25.23[b]	34.41[a]	28.1[ab]	18.98[a]	17.62[a]	24.52[a]
2iP	19.8	16.3[c]	28.5[a]	21.55[b]	17.43[b]	23.77[a]	19.41[ab]	13.11[a]	12.17[a]	16.94[a]
IAA	35.3	29.0[c]	50.7[a]	38.41[a]	31.08[b]	42.39[b]	34.60[ab]	23.38[a]	21.70[a]	30.2[a]
TRP	12.2	9.92[c]	17.5[a]	13.23[a]	10.70[b]	14.60[a]	11.92[ab]	8.053[a]	7.48[a]	10.4[a]
ABA	35.0	28.7[c]	50.2[a]	38.04[b]	30.78[b]	41.97[a]	34.27[ab]	23.15[a]	21.49[a]	29.90[a]

[1]ADE = adenine; ADO = adenosine; DHZ = dihydrozeatin; ZEA = Zeatin; 2iP = isopentenyladenine; TRP = tryptamine; IAA = indoleacetic acid; ABA = abscisic acid.

decreases, accumulation of ethylene and the other phytohormones (auxin, cytokinins, and ABA) is unclear. We suggest that the elevated levels of ethylene may be due to a synergistic effect of elevated levels of IAA and cytokininlike compounds in the presence of TDZ (Lau and Yang, 1973). Acetylsalicylic acid could act by reducing the levels of auxin and cytokininlike compounds, thus reducing the levels of ethylene. Alternatively, the fact that ABA levels are elevated significantly by TDZ may imply that elevated ethylene levels are stress related.

The synthesis and activities of ethylene and other phytohormones (auxin and cytokinin) have been reported to be intimately interrelated (Klee and Romano, 1994). For example, auxin inhibition of the apical hook opening in bean seedlings has been shown to be largely mediated by ethylene (Kang et al., 1967): auxin-stimulated ethylene biosynthesis in pea seedlings (Jones and Kende, 1979) and the expression of the ACC synthase gene encoding the controlling enzyme in ethylene biosynthesis in plants (Sato and Theologis, 1989). Like auxins, cytokinins have also been found to stimulate ethylene production (Mattoo and White, 1991), by suppressing the conversion of IAA into IAA conjugates, resulting in higher free IAA levels, which, in turn, increases the ethylene production (Lau and Yang, 1973). On the other hand, SA has been implicated in inactivating auxin via moderate stimulation of IAA-oxidase activity in callus cultures of *Albizzia julibrissin* (Sankhla et al., 1993) as well as inhibiting ethylene biosynthesis in pear suspension cultures (Leslie and Romani, 1986) and potato leaf-derived protoplasts (Perl et al., 1988), probably by blocking the conversion of ACC to ethylene (Roustan et al., 1990). Because ASA has been shown to undergo spontaneous hydrolysis forming SA, it is likely that ASA may be influencing the ethylene levels via auxin metabolism. The interconvergence of hormones is also well illustrated by the axr2 mutant of *Arabidopsis*, which was originally selected for its auxin resistance, but exhibited cross resistance to ethylene and ABA (Wilson et al., 1990).

In conclusion, thidiazuron increased the levels of ethylene accumulating in the headspace of the culture vessels during somatic embryo induction in geranium hypocotyl cultures. The resultant ethylene may inhibit full expression or development of somatic embryos induced by TDZ. Although the mode of action of ethylene is still unclear, it is likely that there is a major interplay of auxins, cytokinins, ABA, and ethylene during TDZ-mediated somatic embryogenesis in geranium.

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References

Beyer, E. M. A potent inhibitor of ethylene action in plants. Plant Physiol. 58:268–271; 1976.

Biddington, N. L. The influence of ethylene in plant tissue culture. Plant Growth Regul. 11:173–187; 1992.

Biddington, N. L.; Sutterland, R. A.; Robinson, H. T. Silver nitrate increases embryo production in anther cultures of Brussel sprouts. Ann. Bot. 62:181–185; 1988.

Gamborg, O. L.; Miller, R. A.; Ojima, K. Nutrient requirements of suspension cultures of soybean root cells. Exp. Cell Res. 50:151–158; 1968.

Gill, R.; Saxena, P. K. Direct somatic embryogenesis and regeneration of plants from seedling explants of peanut (*Arachis hypogaea*): promotive role of thidiazuron. Can. J. Bot. 70:1186–1192; 1992.

Gill, R.; Saxena, P. K. Somatic embryogenesis in *Nicotiana tabacum*: induction by TDZ of direct embryo differentiation in leaf disc cultures. Plant Cell Rep. 12:154–159; 1993.

Hutchinson, M. J.; KrishnaRaj, S.; Saxena, P. K. Morphological and physiological changes during thidiazuron-induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) hypocotyl cultures. Int. J. Plant Sci. 157:440–446; 1996a.

Hutchinson, M. J.; Murch, S. J.; Saxena, P. K. Morphoregulatory role of thidiazuron: evidence of the involvement of endogenous auxin in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *hortorum* Bailey). J. Plant Physiol.; 149:573–579; 1996b.

Hutchinson, M. J.; Saxena, P. K. Acetylsalicylic acid enhances and synchronizes thidiazuron induced somatic embryogenesis in geranium (*Pelargonium* × *hortorum* Bailey) tissue cultures. Plant Cell Rep. 15:512–515; 1996a.

Hutchinson, M. J.; Saxena, P. K. Role of purine metabolism in thidiazuron-induced somatic embryogenesis of geranium (*Pelargonium* × *hortorum* Bailey) hypocotyl cultures. Physiol. Plant.; in press: 1996b.

Jones, J. F.; Kende, H. Auxin-induced ethylene biosynthesis in sub-apical stem sections of etiolated seedlings of *Pisum sativum* L. Planta 146:649–653; 1979.

Kang, B. G.; Yocum, C. S.; Burg, S. P., et al. Ethylene and carbon dioxide mediation of hypocotyl hook-opening response. Science 156:958–959; 1967.

Klee, H. J.; Romano, C. P. The roles of phytohormones in development as studied in transgenic plants. Crit. Rev. Plant Sci. 13:311–324; 1994.

Kochba, J.; Spiegal-Roy, P.; Neumann, H., et al. Stimulation of embryogenesis in *Citrus* ovular callus by ABA, ethephon, CCC and Alar and its suppression by GA. Z. Pflanzenphysiol. 89:427–432; 1978.

Lau, O.; Yang, S. F. Mechanism of a synergistic effect of kinetin on auxin-induced ethylene production. Plant Physiol. 51:1011–1014; 1973.

Leslie, C. A.; Romani, R. J. Salicylic acid: a new inhibitor of ethylene biosynthesis. Plant Cell Rep. 5:144–146; 1986.

Mattoo, A. K.; White, W. B. Regulation of ethylene biosynthesis. In: Mattoo, A. K.; Suttle, J. C., eds. The plant hormone ethylene. Boca Raton, FL: CRC Press; 1991:21–42.

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

Murthy, B. N. S.; Murch, S. J.; Saxena, P. K. Thidiazuron-induced somatic embryogenesis in intact seedlings of peanut (*Arachis hypogaea* L.): endogenous growth regulator levels and significance of cotyledons. Physiol. Plant. 94:268–276; 1995.

Perl, A.; Aviv, D.; Galun, E. Ethylene and in vitro culture of potato: suppression of ethylene generation vastly improved protoplast yield, plating efficiency and transient expression of an alien gene. Plant Cell Rep. 7:403–406; 1988.

Robinson, K. E. P.; Adams, D. O. The role of ethylene in regeneration of *Helianthus annus* (sunflower) plants from callus. Physiol. Plant. 71:151–156; 1987.

Roustan, J. P.; Latche, A.; Fallot, J. Control of carrot somatic embryogenesis by $AgNO_3$, an inhibitor of ethylene action: effect on arginine decarboxylase. Plant Sci. 67:89–95; 1990.

Sankhla, N.; Davis, T. D.; Snakhla, D., et al. Reversal of salicylic acid-induced inhibition of growth and organogenesis by thidiazuron. Plant Physiol. 102:180 (Abstract); 1993.

Sato, T.; Theologis, A. Cloning the mRNA encoding 1-aminocyclopropane-1-carboxylase synthase, the key enzyme for ethylene biosynthesis in plants. Proc. Natl. Acad. Sci. USA 86:6621–6625; 1989.

Serek, M.; Sisler, E. C.; Reid, M. S. Novel gaseous inhibitor of ethylene binding prevents ethylene effects in potted flowering plants. J. Am. Soc. Hortic. Sci. 119:1230–1233; 1994.

Smith, D. L.; Krikorian, A. D. pH control of carrot somatic embryogenesis. In: Nijkamp, H. J. J.; Van Der Plas, L. H. W.; Van Aartrijk, J., eds. Progress in plant cell and molecular biology: current advances in plant science, biotechnology and agriculture. Dordrecht, Netherlands: Kluwer Academic Publishers; 1990:449–456.

Songstad, D. D.; Petracek, P. D.; Sams, C. E., et al. Effect of 1-aminocyclopropane-1-carboxylic acid and aminoethoxyvinylglycine on ethylene emanation and somatic embryogenesis from orchard grass leaf cultures. Plant Cell Rep. 7:677–679; 1989.

Songstad, D. D.; Armstrong, C. L.; Petersen, W. L. $AgNO_3$ increases type 1 callus production from immature embryos of maize inbred B73 and its derivatives. Plant Cell Rep. 9:699–702; 1991.

SAS Institute Inc. SAS users' guide: statistics, Version 6. Cary, NC: SAS Institute; 1995.

Suttle, J. C. Effect of the defoliant thidiazuron on ethylene evolution from *mung bean hypocotyl segments*. Plant Physiol. 75:902–907; 1984.

Vain, P.; Flament, P.; Soudain, P. Role of ethylene in embryogenic callus initiation and regeneration in *Zea mays* L. J. Plant Physiol. 135:537–540; 1989a.

Vain, P.; Yean, H.; Flament, P. Enhancement of production and regeneration of embryogenic type II callus of *Zea mays* L. by $AgNO_3$. Plant Cell Tissue Organ Cult. 18:143–151; 1989b.

Visser, C.; Qureshi, J. A.; Gill, R., et al. Morphoregulatory role of thidiazuron: substitution of auxin and cytokinin requirement for the induction of somatic embryogenesis in geranium hypocotyl cultures. Plant Physiol. 99:1704–1707; 1992.

Wilson, A. K.; Pickett, F. B.; Turner, J. C., et al. A dominant mutation in *Arabidopsis* confers resistance to auxin, ethylene and abscisic acid. Mol. & Gen. Genet. 222:377–383; 1990.

Yang, S. F.; Hoffmann, N. E. Ethylene biosynthesis and its regulation in higher plants. Annu. Rev. Plant Physiol. 35:155–189; 1984.

Yip, W. K.; Yang, S. F. Effect of thidiazuron, a cytokinin-active urea derivative, in cytokinin dependent ethylene production systems. Plant Physiol. 80:515–519; 1986.