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Effects of Foliar and Root Applications of Methanol on the Growth of *Arabidopsis*, Tobacco, and Tomato Plants

Ingrid Ramírez, Fernando Dorta, Valeria Espinoza, Edra Jiménez, Ana Mercado, and Hugo Peña-Cortés*

Centro de Biotecnología "D. Alkalay L.," Universidad Técnica Federico Santa María, Casilla 110-V, Avenida España 1680, Valparaíso, Chile

Abstract

The effects of aqueous methanol solutions applied as a foliar spray or via irrigation were investigated in *Arabidopsis*, tobacco, and tomato plants. Methanol applied to roots leads to phytotoxic damage in all three species tested. Foliar application causes an increase of fresh and dry weight in *Arabidopsis* and tobacco plants, but not in tomato plants. The increase in fresh and dry weight of *Arabidopsis* plants does not correlate with increased levels of soluble sugars, suggesting that increased accumulation of other products is responsible for the differences in the methanol-treated leaves. Foliar application of methanol can induce pectin methylesterase (PME) gene expression in *Arabidopsis* and tomato plants, activating specific PME genes.

Key words: Methanol; *Arabidopsis*; Tobacco; Tomato; Growth plants; Pectin methylesterase; Gene expression

Introduction

Effects of methanol on growth of cuttings of *Vigna radiata* were reported twenty years ago (Bhattacharya and others 1985). Foliar applications of aqueous methanol have been reported to increase yield, accelerate maturity, and reduce drought stress and irrigation requirements in crops grown in arid environments, under elevated temperatures, and in direct sunlight (Nonomura and Benson 1992). A wide range of C_3 crops and ornamental plants increase their growth and yield of fruit or seed after being sprayed with 10–50% methanol. Treatment of cabbage (*Brassica oleracea capitata*) with methanol resulted in an increase of the vegetative fresh weight of approximately 50%. Comparable enhancements of growth of wheat, radish, pea, and tomato have been reported (Devlin and others 1994; Rowe and others 1994).

Results in other C3 plants, however, including peppermint (*Mentha piperita*, Mitchell and others 1994), orange (*Citrus aurrrrantinium*; Idso and others 1995), and cotton (*Gossypium hirsutum*, van Iersel and others 1995), were inconsistent with the find-

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*Corresponding author; e-mail: hugo.pena@usm.cl

ings of Nonomura and Benson (1992). Furthermore, the results of the field studies on which the original reports were based have been difficult to reproduce (Mitchell and others 1994; Fall and Benson 1996). It has been suggested that the reproducibility of plant responses to methanol treatment could be a result of experimental variations such as exposure time, amount of methanol absorbed, tissue morphology, and accumulation of methanol in the root zone (Hemming and others 1995).

The effect of foliar methanol applications on growth is far beyond that expected of a foliar nutrient. The increased growth and yield has been attributed to the action of methanol as an inhibitor of photorespiration (Nonomura and Benson 1992; Fall and Benson 1996). This was supported by failure of C_4 plants to respond to foliar-applied methanol, by the high light requirements for beneficial effects of methanol in C_3 plants, and by the finding that the ratio of sucrose to glycolate metabolite was increased by methanol. However, the mechanism by which methanol may affect growth and water use efficiency remains unknown. Methanol in small quantities is a natural product of plant metabolism (MacDonald and Fall 1993). Methanol has been described as the simplest natural product derived from plants. Field measurements in the United States revealed that forest air contains methanol, with mean concentrations of 6 ppb at night and 11 ppb during the day (Snider and Dawson 1985). The diurnal pattern for volatile organic compounds (VOCs) and methanol suggests that accumulation of these substances is related to photosynthetic processes (Fall and Benson 1996).

Substantial emissions of methanol from leaves of C_3 plants were found using gas chromatographic analysis or direct enzymatic analysis of gas-phase methanol (MacDonald and Fall 1993; Nemececk-Marshall and others 1995). Free methanol contained in the leaf air space exits leaves along with transpired water vapor (Fall and Benson 1996). The magnitude of methanol emission lies between that of other major biogenic VOCs, such as isoprene and monoterpenes (Guenther and others 1995). Although most plants do not emit significant amounts of isoprene or monoterpenes, all C_3 plants tested so far emit substantial amounts of methanol.

A likely source of methanol in leaves is pectin demethylation in the cell walls (Obendorf and others 1990) in a reaction catalyzed by pectin methylesterase and producing methanol as a by-product (Jarvis 1984). It is likely that pectin demethylation in plant cell walls is the major source of most of the methanol in the atmosphere (Fall and Benson 1996). Young leaves emit much more methanol than fully expanded leaves, sometimes at rates higher than 40 μg (methanol C) h^{-1} g dry wt^{-1} (Nemecek-Marshall and others 1995). These results might be explained by higher rates of pectin demethylation being required during leaf expansion, a period of rapid cell wall synthesis, followed by declining demethylation and methanol production in older leaves. However, a direct correlation between pectin methylesterase activity and methanol release from leaves has not been demonstrated.

This study was designed to provide partial answers to four questions: (1) Does application of methanol affect the development or growth of *Arabidopsis*, tobacco, and tomato plants under controlled growth conditions? (2) How does the effect vary with the method of application? (3) Does the effect vary with the species? (4) Does methanol application alter sugar levels, photosynthetic activity, or gene expression of pectin methylesterase?

Material and Methods

Plant Material and Growth Conditions

Wild-type *Arabidopsis thaliana* (cv. Columbia), tomato (*Lycopersicon esculentum*, cv. Moneymaker), and tobacco (*Nicotiana tabacum*, cv. Samsun) plants were grown from seeds. Most plants were maintained in an indoor growth chamber under controlled conditions (10 h, 25°C day/14 h, 18°C night, 70% relative humidity, supplied with an instantaneous photosynthetic photon flux density of 300 mmol m^{-2} s^{-1} over a 12-h photoperiod). Plants were fertilized by saturating the soil with 1:4 diluted Hoagland's solution approximately every 2 weeks. Control plants were placed in the same chamber as the methanol-treated plants and, in addition, in a separate chamber to avoid any effect from the methanol-treated plants. One chamber was used for each of the three species, and a fourth chamber contained control plants of all three species. The pots of the different treatments were randomized in each chamber.

Methanol Treatment

Spray Application. Before methanol application, the soil of the pots was covered with a plastic membrane to prevent draining of methanol into the

soil. Treatment of the plants was done outside the chambers to avoid spraying other plants. Aqueous methanol solution (2%, 5%, or 10% v/v) was sprayed on the plants with a manual sprayer at a rate of 20 ml per pot, with the nozzle approximately 20 cm above the leaf surface. Each treatment included 10 pots containing five 4-week-old *Arabidopsis* plants, one 6-week-old tobacco plant, or one 6-week-old tomato plant. The same number of control plants was sprayed with water only under the same conditions. Plants were sprayed twice each week, the second spraying 3 days after the first.

Irrigation. The plants used and growth conditions were similar to those described for spray application. The methanol solution was applied directly to the soil, avoiding contact of the solution with the aerial part of the plants. The methanol solution was applied at a rate of 5 ml per *Arabidopsis* pot and 10 ml per tobacco or tomato pot twice each week, the second application 3 days after the first.

''Short Time-course''. *Arabidopsis* and tomato plants were treated one time with 2%, 5%, or 10% (v/v) methanol as described above under *Spray Application*. Samples were collected 2, 6, 18, and 32 h post application and immediately frozen with liquid nitrogen and stored at −80°C.

''Long Time-course''. Plants were sprayed twice each week, the second spraying 3 days after the first, for 6 weeks, and leaf material was harvested after 2, 3, 4, and 6 weeks of treatment. The material was always harvested 40 h after the last methanol application and immediately frozen with liquid nitrogen and stored at −80°C.

Sugar Determination

Leaf tissue was harvested, weighed, and immediately frozen in liquid nitrogen. Between 0.5 and 1 g of frozen leaf tissue was homogenized in a chilled mortar in 0.25 g polyvinylpyrrolidone (PVP) and 2 ml of 50 mM sodium phosphate buffer at 4°C. The homogenate was centrifuged at 16,000 × g for 10 min. The resulting supernatant was desalted using pre-packed Sephadex G-25 M PD 10 columns (Pharmacia Biotech AB, Uppsala, Sweden), and soluble sugars such as glucose, fructose, and sucrose were measured using a UV-Test Commercial Kit (Boehringer Mannheim, Germany) according to the instructions of the provider. Student *t*-tests were performed using MS Excel 2003 (Microsoft Corporation, Seattle, WA, USA). The term significant is used to indicate differences for which $p < 0.05$.

Growth of Plant Roots on Medium Containing Methanol.

Arabidopsis, tobacco, and tomato plants were grown under tissue culture conditions. Germinated *Arabidopsis* seeds and tobacco and tomato plants were kept under a 16 h light/8 h dark period on Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose at 22°C, either without methanol or supplemented with different methanol concentrations. The development of *Arabidopsis* roots was observed after 12 days, whereas the tobacco and tomato root development was observed after 19 days.

RNA Isolation and Gene Expression Analysis

Total RNA was prepared from green leaf tissue removed from control plants and methanol-treated plants at the indicated times after methanol application. Two to three leaves were taken per plant from each pot and immediately frozen in liquid nitrogen. The fresh tissue was then rapidly ground in liquid nitrogen and 1.5 ml Trizol reagent (Life Technologies) was added to 200 mg of ground material. The manufacturer's instructions for the Trizol RNA extraction method were followed.

Total RNA (10 μg per sample) was loaded on 1.5% agarose formaldehyde gels. After electrophoresis, the gels were blotted to nylon membranes (Hybond NX, Amersham) by capillary action in 20 × SSC (175.3 g/l NaCl, 88.2 g/l trisodium citrate, pH 7.0; Sambrook and others 1989). The air-dried membrane was then UV irradiated in a linker BLX254 (Vilber Lourmat Biotechnology, Marne la Vallée, France). Purified cDNA fragments corresponding to genes of pectin methylesterase were labeled directly with [a-^{32}P]dCTP using the Prime-a-gene labeling system (Promega, Madison, Wisconsin, USA) according to the manufacturer's instructions. Labeled probes were allowed to hybridize for a minimum of 12 h to membrane cross-linked RNA equilibrated in buffer as described by Harms and others (1995). Membranes were then washed twice for 30 min in 2 × SSC, 0.1% (v/v) SDS at 65°C, and scanned with a Personal Molecular Imager FX (BioRad, Carlsbad, California, USA). The genes used for analysis of PME gene expression in *Arabidopsis* plants were *AtPme1* (NM-104433), *AtPme2* (NM-120004), and *AtPme3* (NM-118656), obtained from the Tair *Arabidopsis* stock center (http://www.*Arabidopsis*.org). For analysis of tomato PME gene expression, two potato PME genes were used (Pilling and others 2004). *Pest1* shows a 93% identity to

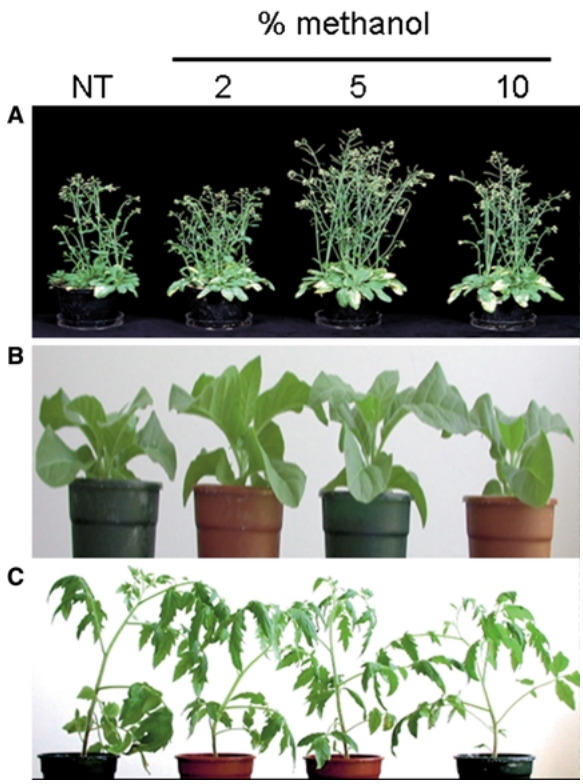

Figure 1. Effect of foliar methanol application on the growth of *Arabidopsis*, tobacco, and tomato plants. Wild-type *Arabidopsis thaliana* (**A**), tobacco (**B**), and tomato (**C**) plants grown in growth chambers under controlled conditions (10 h, 25°C day/14 h, 18°C night, 70% relative humidity, supplied with a instantaneous photosynthetic photon flux density of 300 mmol m^{-2} s^{-1} over a 12 h photoperiod) were treated with methanol as described in *Material and Methods*. Control plants (NT) were treated with water only. The figure shows the average of results obtained from four independent experiments with *Arabidopsis* plants treated for 3 weeks and tobacco and tomato plants treated for 6 weeks.

the tomato PME gene (LEU70675) expressed in fruits, whereas *Pest2* has a 90% identity to the tomato PME (LEU70676) expressed in leaves. *Pest1* (AF152171) and *Pest2* (AF152172) were a gift of Dr. J. Fisahn (Max-Planck Institute for Plant Molecular Physiology, Golm, Germany). A cDNA encoding 18SrRNA was used as a control for the amount of RNA loaded on the gel

Results

The Method of Methanol Application Affects the Growth of Plants Differently

A preliminary dose–response study was performed to determine whether methanol would produce tissue injury in any of the species under investigation at the concentrations that were expected to be used. No other compounds, such as urea, Fe EDTA or wetting agents (Nonomura and Benson 1992; Mortensen 1995), were included in the spray formulation so that the effect of methanol could be examined without confounding effects. *Arabidopsis*, tomato, or tobacco plants were treated with different aqueous solutions of methanol from 0.5% to 40 % (v/v) for 3 or 6 weeks as described in *Material and Methods*. No leaf toxicity or necrosis was observed with any of the crops when aqueous methanol was applied at concentrations below 15% (v/v). Necrotic injuries were observed in tomato and tobacco leaves at concentrations higher than 15%, whereas *Arabidopsis* plants showed damage at methanol concentrations higher than 30% after 2 weeks of treatment. We decided to use concentrations of methanol between 2% and 10% (v/v) for further studies.

Figure 1 shows the results obtained after spraying methanol at three different concentrations (2%, 5%, and 10% v/v) on *Arabidopsis* (A), tobacco (B), and tomato (C) plants in four independent experiments. We consistently found that, with *Arabidopsis*, visual differences among the treatments could be observed after 3 weeks of treatment. Plants treated with aqueous methanol solution of 5% (v/v) have bigger leaves and more flowers than the non-treated control plants. No visual increase was observed in the plants treated with methanol 2% (v/v). Leaf chlorosis was observed after treatment with 10% methanol, but not with 2% or 5% (v/v). The damage to *Arabidopsis* leaves after application of 10% methanol is detectable only after 3 weeks of treatment.

Tobacco plants treated for 6 weeks with methanol showed visual differences, whereas no differences were observed in tomato plants treated under the same conditions. None of the methanol solutions (2%, 5%, or 10%) caused damage to tobacco plants. Methanol at 2% and 5% (v/v) promoted the growth of tobacco plants, whereas plants treated with 10% (v/v) methanol grew in much the same way as control non-treated plants (Figure 1B). Tobacco plants treated with 10% (v/v) methanol showed no damage even after treatment for as long 10 weeks (data not shown).

Tomato plants treated with methanol showed no injuries as a result, and no visual differences were observed after 3 weeks of treatment (Figure 1C), or at even longer periods (data not shown).

Completely different results were obtained with all three species when methanol solution was applied directly to the soil (Figure 2). Clear visual differences could be observed between the treated and non-treated control plants. All three concen-

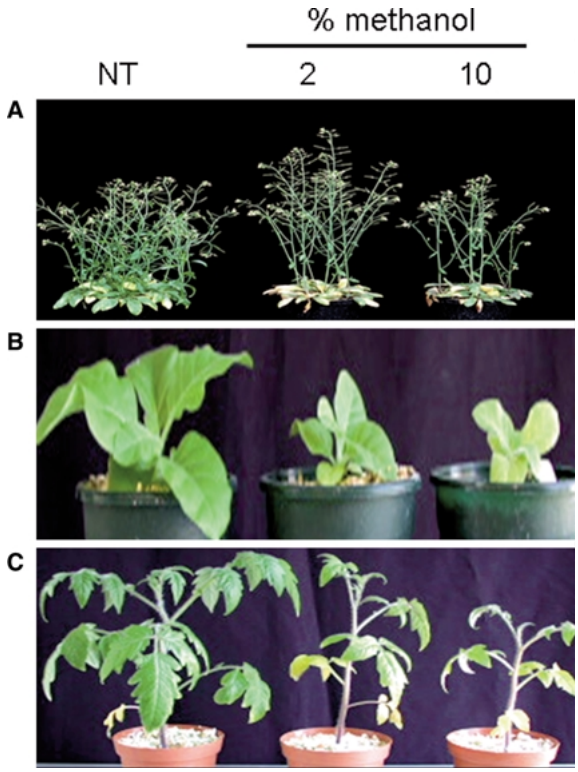

Figure 2. Effect of methanol application to roots on growth of *Arabidopsis*, tobacco, and tomato plants. *Arabidopsis thaliana* (**A**), tobacco (**B**), and tomato (**C**) plants grown under the same conditions as described for Figure 1 were treated with 2% or 10% (v/v) methanol. Alcohol solutions were applied to the soil twice a week. Control plants (NT) were treated with water only. The results shown are the average results of four independent experiments obtained with *Arabidopsis* plants treated for 3 weeks and tobacco and tomato plants treated for 4 weeks.

trations of methanol caused severe plant damage and reduced growth of the plants. A reduction in root development and growth was also observed in the treated plants (data not shown). The effect could be observed in all three species after the second week of treatment. *Arabidopsis* plants died after 5 weeks of treatment, and tobacco or tomato plants died after 8 weeks.

To quantify the differences observed in the two experiments (spray application and irrigation), plants were collected at the end of the experiments and the fresh and dry weights were determined (Figures 3 and 4). Foliar application of methanol solutions resulted in significant growth stimulation in *Arabidopsis* and tobacco plants, but not in tomato. In both *Arabidopsis* and tobacco, 2% and 5 % (v/v) methanol produced an increase in both fresh and dry weight, with 5% methanol having a greater effect. Treated *Arabidopsis* plants showed a fresh weight of around 18 g, whereas the fresh weight of control plants averaged 11 g, an increase of approximately 60% (Figure 3A). The treated plants had a dry weight that was approximately 50% higher than that of the control plants (Figure 3B). Similar results were obtained with tobacco plants. Treatment with 5% methanol had the greatest effect on fresh weight (Figure 3C) and dry weight (Figure 3D) of the plants. The increase is less than that observed in *Arabidopsis*, but treated plants showed an increase of both fresh and dry weight close to 30%. In contrast, determination of fresh and dry weights confirmed that foliar application of methanol solutions did not affect the growth of tomato plants (Figure 3E and 3F).

Irrigation with methanol solutions caused a strong reduction of leaf and stem fresh and dry weights, with the maximum effect at the highest concentration tested in all three species (Figure 4). Even lower concentrations (0.5%) caused a toxic effect in *Arabidopsis*, tobacco, or tomato plants (data not shown), affecting growth and development of plant roots. Figure 5 shows that methanol inhibits root development in *Arabidopsis*, tobacco, and tomato plants *in vitro*. An inhibitory effect is already apparent at a concentration of 0.5% (v/v), and at higher methanol concentrations like 5% (v/v) the plants are unable to form roots.

The results show that the effect of methanol is different depending on the plant species and also depending on the mode of application to the plant.

Methanol Application Affects Sugar Metabolism

Plants can use foliar-applied methanol as a C source and it is known that ^{14}C-labeled methanol is readily assimilated by plant cells, with formation of $^{14}CO_2$ by an oxidation process via formaldehyde and formate (Nonomura and Benson 1992; Fall and Benson 1996; Fall 2003). Cossins (1964) reported that some of the C from methanol was incorporated into sugars and amino acids. To determine if the differences in the effects of foliar application of methanol in *Arabidopsis*, tobacco and tomato plants are due to differences in the effects of methanol on plant metabolism related to generation of carbohydrates, we decided to evaluate the content of glucose, fructose, and sucrose in the leaves harvested from plants treated with 2%, 5%, or 10% (v/v) methanol. Leaf material of *Arabidopsis* and tomato plants from the treatments described in Figure 1 (A and C) were used for determination of the total pool size of sugars. *Arabidopsis* leaves were harvested from the plants treated for three weeks with

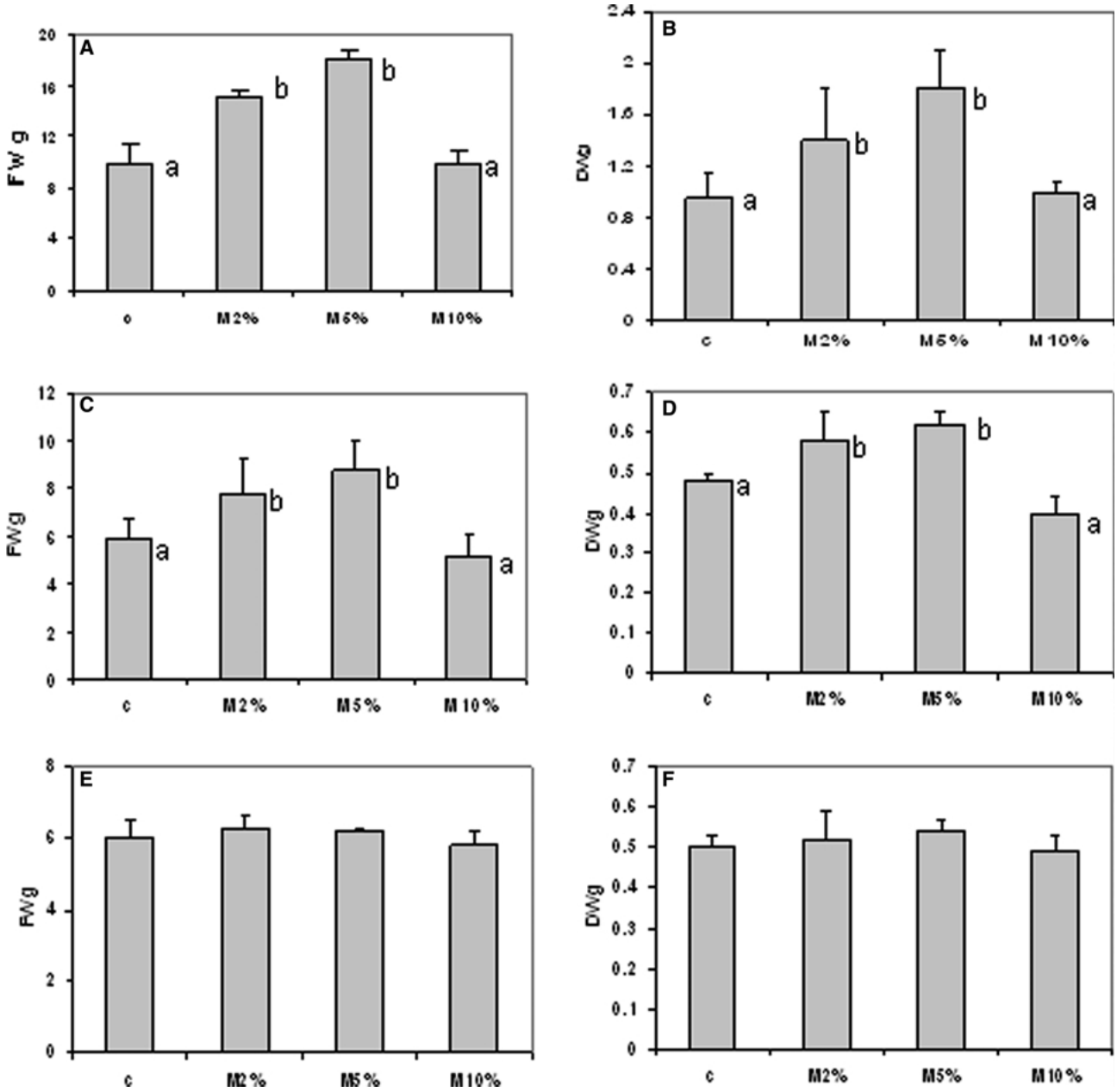

Figure 3. Fresh and dry weight in plants following methanol treatment. Leaf material from plants treated as described for Figure 1 were harvested at the end of the experiment, and fresh and dry weights were determined. Fresh weights of *Arabidopsis* (**A**), tobacco (**C**), and tomato (**E**) were determined after 3 weeks (*Arabidopsis*) and after 6 weeks (tobacco and tomato) post-treatment, respectively. The plants were subsequently dried at 70°C for 20 h, and the dry weights of *Arabidopsis* (**B**), tobacco (**D**), and tomato (**F**) were determined. Means followed by the same letter are not significantly different from each other, whereas different letters indicate significant differences (*t*-test, $p \leq 0.05$). Fresh and dry weight values are mean ± SD ($n = 30$).

methanol and tomato leaves were obtained from plants treated for 6 weeks. All leaves were harvested at noon to minimize diurnal effects on sugar metabolism. Figure 6 shows that neither *Arabidopsis* (A) nor tomato (B) plants treated with methanol solutions had differences in sugar content compared to control plants at any methanol concentration tested. These results suggest that the increase in growth (fresh and dry weight) in *Arabidopsis* plants following methanol treatment is not related to an increase in sugar content.

To examine more closely the effect of foliar application of methanol on sugar content in plants, *Arabidopsis* and tomato plants were treated with

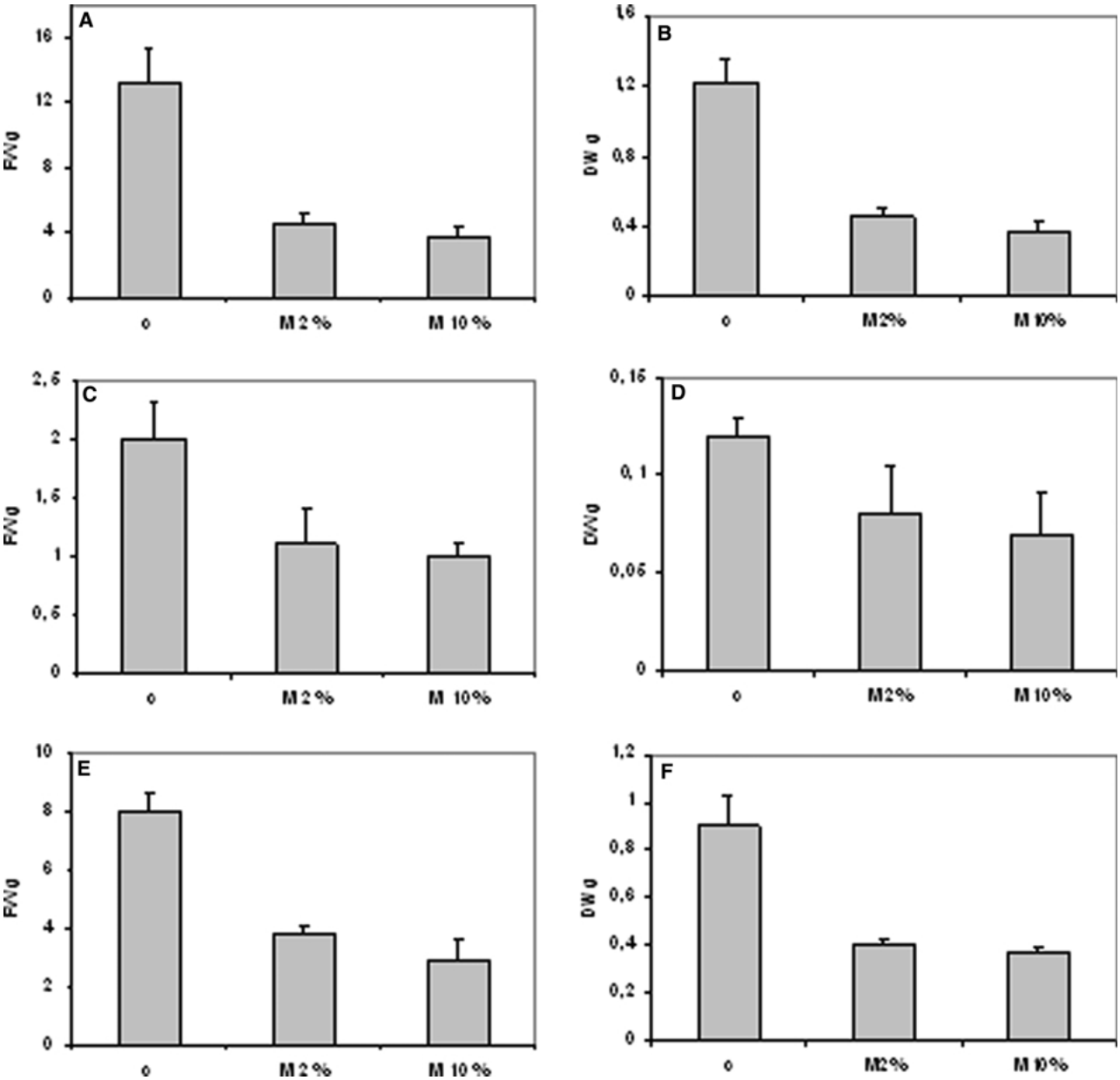

Figure 4. Effects of application of methanol to roots on fresh and dry weight. Leaves from plants treated as described for Figure 2 were harvested at the end of the experiment and fresh and dry weights were determined. Fresh weights of *Arabidopsis* (**A**), tobacco (**C**), and tomato (**E**) were determined after 3 weeks (*Arabidopsis*) and after 4 weeks (tobacco and tomato) post-treatment, respectively. The plants were subsequently dried at 70°C for 20 h, and the dry weights of *Arabidopsis* (**B**), tobacco (**D**), and tomato (**F**) were determined. Means followed by the same letter are not significantly different from each other, whereas different letters indicate significant differences (*t*-test, $p < 0.05$). Fresh and dry weight values are means ± SD ($n = 30$).

methanol solutions one time as described in *Material and Methods* (*Short Time Course*). Figure 7 shows that methanol has different effects on the content of sugar in *Arabidopsis* (A) and tomato (B) plants in the first hours post-application. In *Arabidopsis*, there is a slight increase in glucose and fructose between 6 and 18 h post-treatment, whereas a marked decrease in sucrose is observed following treatment with all three methanol concentrations tested (Figure 7A). Tomato plants did not show significant changes in glucose, fructose, or sucrose in response to 2%, 5%, or 10% methanol (Figure 7B). Similarly, tobacco plants treated with 2%, 5%, or 10% methanol did not show changes in basal contents of

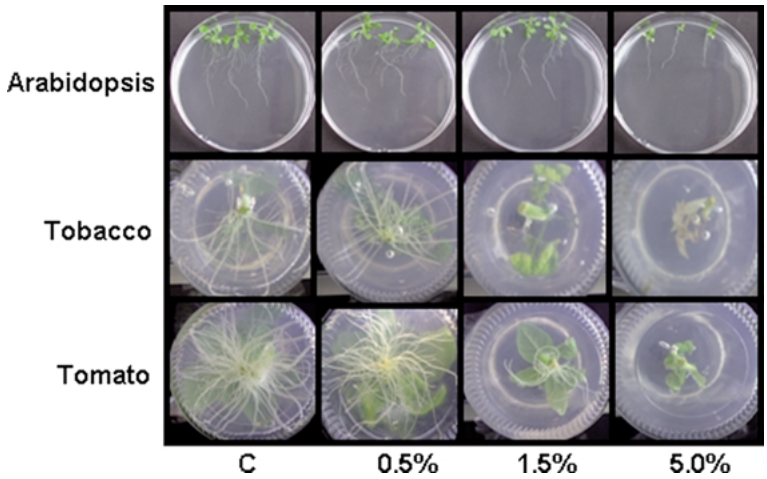

Figure 5. Effect of methanol on root growth *in vitro*. *Arabidopsis* seeds were germinated by the same tissue culture conditions as tobacco and tomato plants were grown. To this, the plants were kept under a 16 h light/8 h dark period on Murashige and Skoog medium (Murashige and Skoog 1962) supplemented with 2% (w/v) sucrose at 22°C. Young *Arabidopsis* plants or apical meristems of tobacco and tomato plants were cultivated either on Murashige and Skoog medium containing 2% (w/v) sucrose (**C**) or on the same medium supplemented with different methanol concentrations (0.5%, 1.5%, and 5%). Root formation in *Arabidopsis* was observed 12 days after treatment, whereas tobacco and tomato roots were observed after 19 days. The figure represents the average situation observed in three independent experiments.

glucose, fructose, or sucrose compared to those observed in untreated ones (data not shown).

Methanol-induced PME Gene Expression in *Arabidopsis* and Tomato Plants

Methanol is emitted by actively growing plant tissues (Nemecek-Marshall and others 1995) and ripening fruits (Frenkel and others 1998). Genetic evidence suggests that the production of methanol in tomato fruit is regulated by pectin methylesterase (Frenkel and others 1998). The pectin methylesterase (PME) reaction is a simple esterase reaction that forms a galacturonic acid side chain on the homogalacturonic acid (HGalA) backbone and methanol as the other product. To gain a broader insight into the effect of methanol on PME gene expression, *Arabidopsis* and tomato plants were treated with foliar applications of methanol at three concentrations (2%, 5%, and 10%) under different regimens described as ''Short Time Course'' and ''Long Time Course'' in *Material and Methods*. The expression of three *Arabidopsis* (*AtPme1*, *AtPme2* and *AtPme3*) and tomato *PME* genes was tested following the two kinds of treatments. For the analysis of tomato PME expression, two *PME* genes isolated from potato (*Pest1* and *Pest2*) were used (Pilling and others 2004). Figure 8 shows the results obtained in the ''short time course'' experiments. Both *Arabidopsis* and tomato plants responded to methanol application by accumulating at least one form of PME transcript within 2 h of treatment. In *Arabidopsis*, the application of 2%, 5%, or 10% (v/v) methanol lead to the accumulation of *AtPme1* transcripts only, whereas no accumulation of *AtPme2* or *AtPme3* was detected in any treatment (Figure 8A). *AtPme1* gene activation occurred between 2 and 18 h post treatment, and no effect was observed 32 h after application of 2%, 5%, or 10% methanol. There were no differences between *AtPem1* transcript levels with the three methanol concentrations.

In tomato plants, the application of methanol 2%, 5%, or 10% (v/v) promoted the accumulation of only the tomato homolog to potato *Pest2* gene, and none of the treatments altered the expression of the tomato homolog to the potato *Pest1* gene (Figure 8B). The activation of the tomato homolog to *Pest2* increased with time post-treatment, the most pronounced accumulation being between 18 and 32 h after application. The highest levels of transcripts were observed following application of 5% methanol. As with *Arabidopsis*, the tomato plants respond to methanol by activating *PME* genes within 2 h after methanol application.

The ''long time course'' experiment (Figure 9) shows that methanol applied twice a week for several weeks affects the expression of PME in both *Arabidopsis* (Figure 9A) and tomato (Figure 9B) plants. This treatment induces the same forms of PME induced in the ''short time course'' experiment (Figure 8A and B).

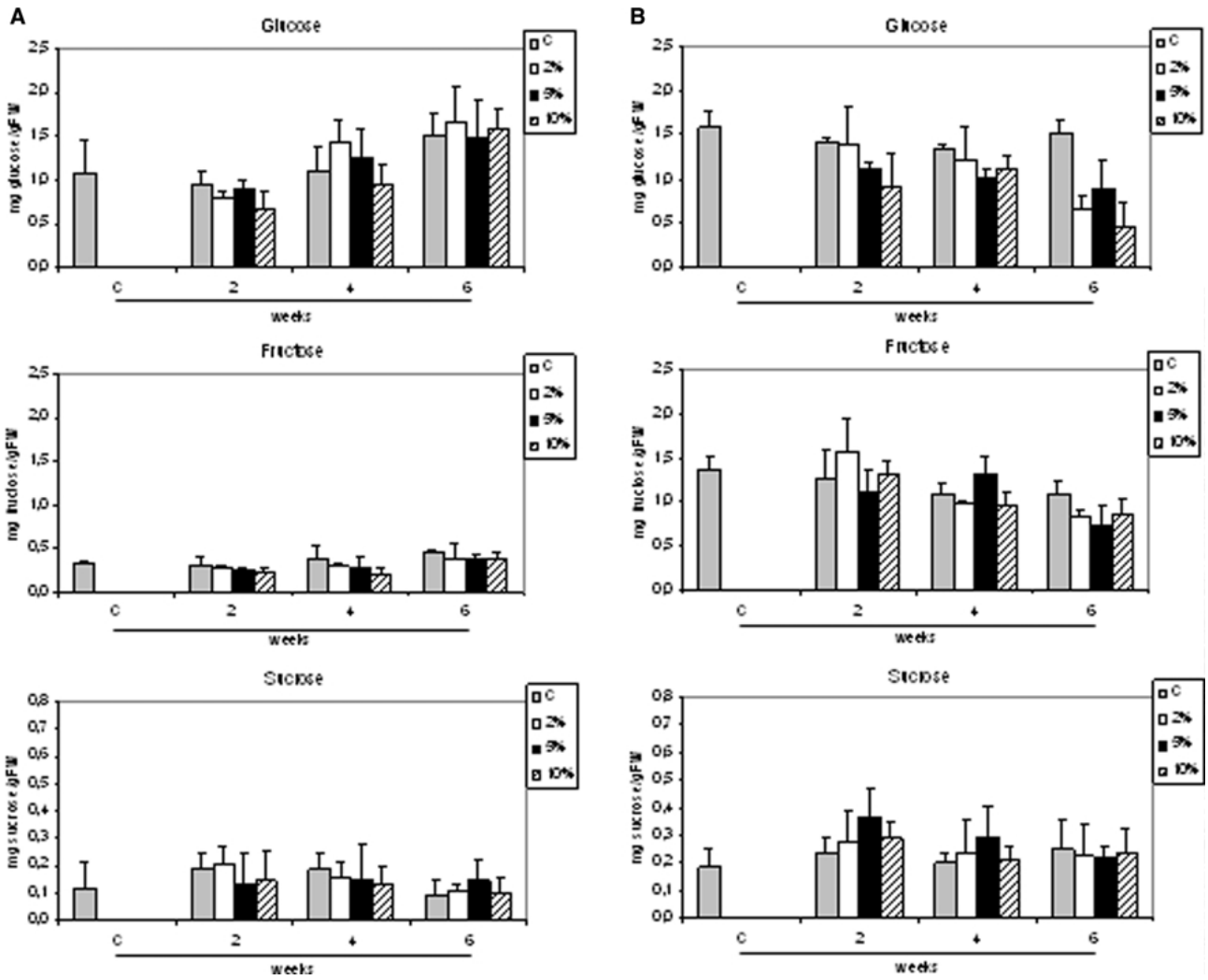

Figure 6. Effects of periodic foliar methanol applications on soluble sugar levels. Leaves from *Arabidopsis* and tomato plants treated as described for Figure 1 were used for sugar measurements as described in Material and Methods. Glucose, fructose, and sucrose were determined in *Arabidopsis* (**A**) and tomato (**B**) leaves. Glucose, fructose, and sucrose contents are mean ± SD (n = 20).

Discussion

Since the early 1990s, several publications have reported dramatic effects of foliar methanol applications on growth, yield, and water-use efficiency of a number of diverse plant species, including increases of 25–50% in thickness of tomato leaves and stems, maximum weight of heads of Savoy cabbage, fruit yield in watermelon, length and width of wheat leaves, and vegetative growth in barley (Nonomura and Benson 1992; Albrecht and others 1995; Fall and Benson 1996; Fall 2003). However, the effects of methanol on growth in field studies have been quite variable and difficult to reproduce (Hartz and others 1994; Mitchell and others 1994; Hemming and others 1995).

Prior investigations have found that at concentrations of 10% of ethanol and methanol were deleterious to tomato when applied to roots, whereas aerial parts withstood higher concentrations (Rowe and others 1994). In the present study, in all three species tested, methanol applied to the roots caused a yield decrease and significant injury leading to early death of the plants, with significant damage occurring even with solutions of 2% methanol. The large differences observed in the effects of applications of methanol solutions to the foliage and to the roots may contribute to the variability in the results of field studies if the amount of methanol reaching the roots varies with different modes of application.

In most studies only morphological observations or basic parameters have been reported, and there is

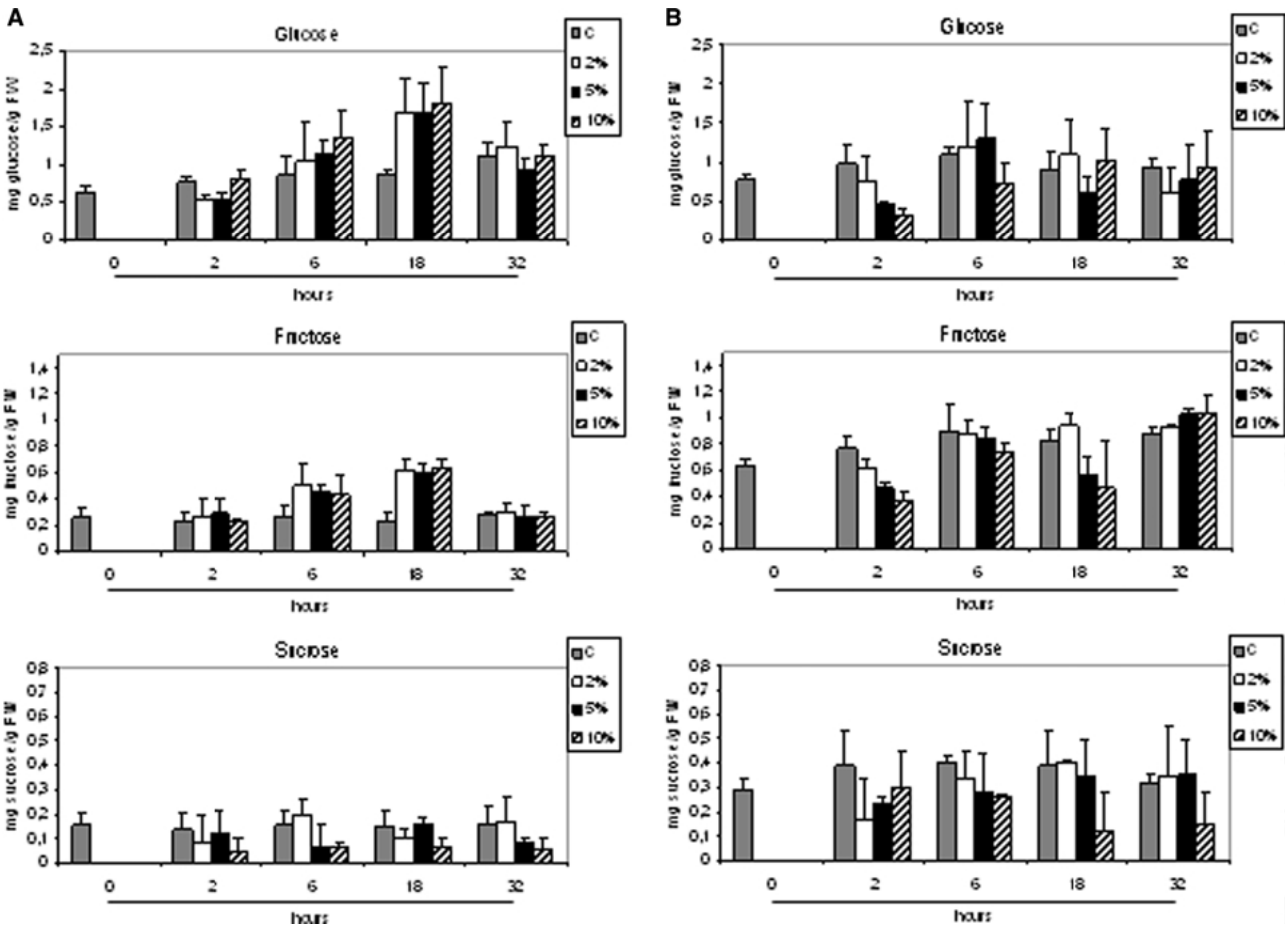

Figure 7. Soluble sugar levels in *Arabidopsis* and tomato plants following a single foliar application of methanol. *Arabidopsis* and tomato plants were treated with different methanol concentrations (2%, 5%, or 10%) once, and samples were harvested 2, 6, 18, and 32 h after the application. Soluble sugar measurement was performed as described in *Material and Methods*. Glucose, fructose, and sucrose levels were determined in *Arabidopsis* (**A**) and tomato (**B**) leaves. Each glucose, fructose, and sucrose determination is the mean ± SD (n = 20).

very limited information that is applicable to development of an understanding of the mechanisms underlying the effects of methanol on growth. In the present study we investigated the effect of application of methanol solutions to plants grown under controlled conditions in growth chambers to establish a system for investigation of the mechanism underlying the response.

The results of foliar applications of methanol solution under controlled conditions in this study confirm prior observations reporting an increase of growth and yield in tobacco (Nonomura and Benson 1992) and lack of a significant effect in tomato plants (Hartz and others 1994; McGiffen and others 1995). Foliar application of methanol solutions to *Arabidopsis* plants resulted in significant increases in fresh and dry weight, indicating that this widely used model plant can be useful in the investigation of the molecular mechanisms underlying the response to methanol.

Plants can use foliar-applied methanol as a C source and it is known that ^{14}C-labeled methanol is readily assimilated by plant cells with formation of $^{14}CO_2$ by an oxidation process via formaldehyde and formate (Nonomura and Benson 1992; Fall and Benson 1996; Fall 2003). Cossins (1964) showed that methanol is mainly converted to CO_2, which probably can be used as a source of carbon in plants. Because prolonged foliar methanol application resulted in an increase in fresh and dry weight in both *Arabidopsis* and tobacco plants but not in tomato, the total pool sizes of glucose, fructose, and sucrose were determined in leaves harvested from *Arabidopsis* plants showing the phenotype (Figure 1A) and in tomato plants, which did not show differences compared to control plants. The content of all three sugar types measured (glucose, fructose, and sucrose) was not affected in *Arabidopsis* or in tomato plants following methanol treatment under such conditions. The total sugar content was

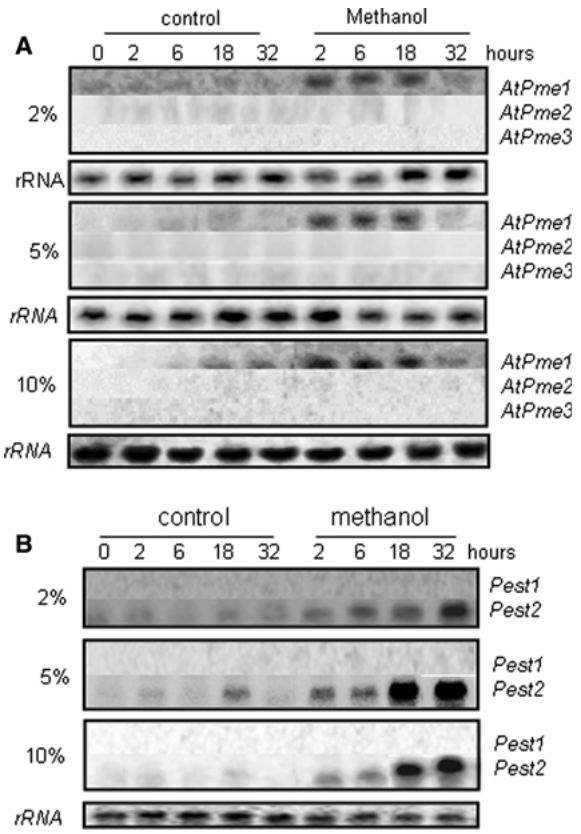

Figure 8. Effect of methanol on PME gene expression. Total RNA was isolated from leaves of control (sprayed with water only) or treated (sprayed with 2%, 5%, or 10% methanol) plants described for Figure 6 and in *Material and Methods*. Samples from *Arabidopsis* (**A**) and tomato plants (**B**) were collected 2, 6, 18, and 32 h after water or methanol application. Control (0) represents a sample of non-treated plants before water application. The experiment was repeated four times with similar results. The autoradiogram shows the results of an RNA gel blot hybridization of total RNA (10 μg per line) against radioactive *AtPme1*, *AtPme2*, and *AtPme3* (**A**) or *Pest1* and *Pest2* (**B**) cDNA probes. To verify the amount of RNA loaded onto the gel, a radioactive fragment encoding an 18SrRNA was used.

similar to that in control plants, so the increased fresh and dry weight must have been due to the accumulation of other substances.

Analysis of sugar content in leaves harvested hours after methanol application demonstrated that methanol application had no effect on sugar content in tomato leaves as well. Contrary to this possibility, leaves of *Arabidopsis* treated with methanol showed an increase of glucose and fructose and a decrease of sucrose hours after methanol application. A similar soluble sugar behavior (increase of glucose and fructose and a decrease of sucrose) has also been observed in leaf development in *Arabidopsis* leaves, and it might be involved in mediating alterations in the expression of photosynthetic genes and/or senescence-associated genes during leaf development (Stessman and others 2002). Moore and others (1999) suggested that the cycling of sucrose synthesis and hydrolysis through invertase and hexokinase provides a mechanism for the generation of hexose signals to inhibit photosynthetic gene expression by a feedback mechanism. Downie and others (2004) reported recently the gene expression analysis in response to methanol stimulation in leaves of *Arabidopsis thaliana* harvested 1, 24, and 72 h after methanol treatment. Interestingly, based on functional category analysis of these genes, at 1 h, the largest upregulated categories were metabolism, cell communication/signal transduction processes, defense genes associated with transcription, and aspects of RNA processing; a few related to cell division and growth, but none were associated with photosynthesis. Based on this result, one could assume that methanol may inhibit the expression of genes associated with the photosynthetic process in *Arabidopsis* plants by increasing the hexose pools sizes. On the one hand, the increase in glucose and fructose in *Arabidopsis* leaves hours after methanol application supports the functionality of a methanol metabolism pathway to provide the CO_2 molecules for sugar formation (Cossins 1964). On the other hand, Gout and others (2000) demonstrated that methanol readily entered into sycamore (*Acer pseudoplatanus* L.) cells to be slowly metabolized to serine, methionine, and phosphatidylcholine. These results suggest that methanol is oxidized to formate, which is the potential single-carbon source in higher plants. Whether this methanol metabolism pathway (formate) or oxidation to CO_2 is operating in the leaves of plants remains unknown.

Most plants produce and emit methanol, especially during the early stages of leaf expansion because of pectin demethylation by the action of PME. This enzyme is a cell-wall associated protein that demethoxylates pectin to form carboxylated pectin while releasing methanol and a proton (Harriman and others 1991; Fall 2003). Although methanol production is correlated with PME activity in germinating seeds or other plant tissues (Nemecek-Marshall 1995), the role of PME in methanol accumulation in plants, as well as the effect of methanol on *PME* gene expression, has not been established. The results obtained in this study demonstrate that methanol application affects the *PME* gene expression differently in *Arabidopsis* and tomato plants. Methanol-induced *PME* gene expression seems to be specific for certain *PME*

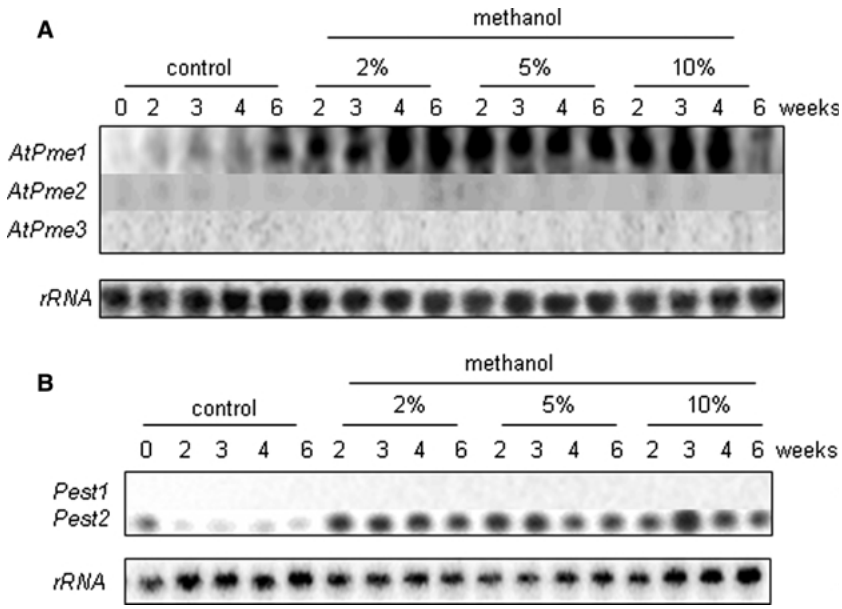

Figure 9. *PME* gene expression in *Arabidopsis* and tomato plants following methanol application. Total RNA was isolated from leaves of control (sprayed with water only) or treated (sprayed with 2%, 5%, or 10% methanol) plants described for Figures 1 and 6 and in *Material and Methods*. Samples were taken from *Arabidopsis* (**A**) and tomato plants (**B**) 2, 3, 4, and 6 weeks after either the initiation of water or methanol application twice a week. Control (0) represents a sample of non-treated plants before water application. The experiment was repeated four times with similar results. The Northern blot analysis was performed as described in the legend for Figure 8.

genes in both *Arabidopsis* and tomato plants. In *Arabidopsis*, only the expression of the *AtPem1* gene is affected by methanol application at concentrations between 2% and 10% (v/v). The activation of this gene occurs within hours after application in a transient manner (between 2 and 18 hours after treatment), whereas prolonged methanol application leads to a permanent expression of this gene. Neither of the other forms (*AtPme2* or *AtPme3*) were affected by methanol treatment. Similar results were obtained in the experiments using tomato plants. The spatial distribution of *Pest2* mRNA resembled the gene expression pattern of tomato *pmu1*, which is ubiquitously expressed according to Gaffe and others (1997). The tomato *PME* gene, which is normally expressed in the leaves, is up-regulated following methanol treatment with concentrations between 2% and 10% (v/v). The activation of this gene is slower than *AtPme1* and does not show the transient behavior. Accumulation of transcript 32 h after the methanol application is also observed in the experiments where methanol was applied over a period of weeks. The tomato *PME* gene, highly homologous to *Pest1*, which is normally expressed in the fruits, is not activated in tomato leaves by methanol application under any condition. The results suggest that methanol can induce *PME* gene expression in *Arabidopsis* and tomato plants in a specific manner, and that the *AtPme1* and *Pest2* genes should contain some common regulatory element allowing them to respond to this alcohol at the concentrations tested. The failure to induce the other *PME* genes in both *Arabidopsis* and tomato by foliar methanol application suggests either that these genes do not contain such regulatory units able to respond to methanol or that other factors are required for their activation. Some transgenic approaches have demonstrated that the inhibition of *PME* gene expression in tomato fruits by antisense RNA had a marked influence on fruit pectin metabolism and increased the soluble solids content of fruits, but did not interfere with the ripening process (Tieman and others 1992) or lead to a reduction in methanol content (Frenkel and others 1998).

Inhibition of potato PME by expressing the *Pest2* gene in antisense orientation affected plant development as reflected by smaller stem elongation rates of selected transformants when compared with control plants. It also leads to a reduction in height throughout the entire course of development. Significant differences in leaf growth patterns were detected between wild-type and transgenic plants. The visual phenotypes could be correlated with modifications of ion accumulation and partitioning within the transgenic plants.

This retardation in growth could be correlated with a depletion of total Ca^{2+} in the apical stem segments of the transgenic plants (Pilling and others 2004). Filling intracellular stores with Ca^{2+} in young cells may be a prerequisite for growth. Given the immobile nature of Ca^{2+}, meristem and elongating cells would be at most risk of Ca^{2+} deficiency. The shoot apex receives little Ca^{2+} from what remains in the transpiration stream. Furthermore, intracellular Ca^{2+} is continuously diluted by cell division and cell elongation. Thus one hypothesis is that young growing cells must first fill their intracellular stores to continue elongation (Sze and others 2000). Because PME-inhibited plants were significantly depleted of Ca^{2+} in their apical stem segments, this deficiency could have induced growth retardation. Interestingly, potato plants that constitutively overexpressed PME, displayed the opposite phenotype in that they showed enhanced stem elongation rates during early stages of development (Pilling and others 2000). Our results demonstrated that exogenous methanol application activates *PME* gene expression in both *Arabidopsis* and tomato plants. Methanol treatment also enhances growth in *Arabidopsis* and tobacco plants. Considering our results and those obtained by Frenkel and others (1998), Pilling and others (2000, 2004), as well as the hypothesis put forward by Sze and others (2000), we can state that *PME* gene expression is involved in the mechanisms responsible for plant growth. Methanol-activated gene expression may lead to higher degradation of pectin in the cell wall, producing additional methanol and altering the amount of Ca^{2+} availability. The methanol generated as the result of demethoxylation of pectin catalyzed by PME allows a persistent activation of *PME* gene expression in the plant tissue. The persistent activation of PEM may lead to a continued degradation of pectin and changes in Ca^{2+} accessibility. This amount of available Ca^{2+}, generated as a result of the methanol-activated *PEM* gene expression, may move to young growing cells filling their intracellular stores to continue elongation. Thus, periodical or continued application of methanol to plant leaves may facilitate Ca^{2+} availability, which might be used to promote growth and elongation in plant tissues.

Whether the methanol-induced genes (*AtPme1* or *Pest1*) analyzed in this study are really catalyzing pectin demethylation and producing methanol in the amount necessary to alter the level of Ca^{2+} is still to be determined.

The difference in toxicity of methanol solutions for shoots and roots may be related to the ability to oxidize it. It has been proposed that methanol, in plants and other organisms, can be oxidized, successively, to formaldehyde, formic acid, and CO_2 (Fall and Benson 1996). The intermediate formaldehyde represents a reactive electrophilic species with high toxicity that is rapidly detoxified by a pathway involving three key enzymes: (1) NAD-dependent formaldehyde dehydrogenase (FALD), (2) thiolesterase S-formylglutathione (FGH), and (3) NAD-dependent formate dehydrogenase (FDH), which oxidizes the formate to CO_2 (Haslam and others 2002; Kordic and others 2002; Achkor and others 2003).

Previously, induction of FDH by treatment with 20% methanol suggested that this pathway has the ability to respond to methanol treatment in potato leaves (Hourton-Cabassa and others 1998). The differences observed between experiments in this study (spraying and irrigation of methanol) may be explained by the capacity of the plants to oxidize methanol to CO_2. The increased growth in *Arabidopsis* and tobacco, as well as the ineffectiveness of methanol on tomato plants (without phytotoxic effect) observed after application of methanol by spraying, may be the result of a pathway operating to metabolize the methanol in a way that avoids accumulation of the highly toxic intermediate formaldehyde while allowing formation of CO_2.

Recently, Downie and others (2004) demonstrated that within the functional class metabolism, the genes encoding detoxification proteins represent by far the most strongly regulated group in *Arabidopsis* leaves following foliar methanol application. It is reasonable to think that among such genes could be those responsible for formaldehyde detoxification avoiding the toxic effect of it and allowing the oxidation of methanol to CO_2.

The involvement of certain reactive electrophilic species in diseased and stressed tissues is highly documented (Imbusch and Mueller 2000; Jalloul and others 2002). A range of electrophilic species such as unsaturated carbonyl compounds have been shown to elicit many of the stress responses described in this study (Alméras and others 2003), so the possibility of the oxidation product, formaldehyde, being the active electrophilic elicitor damaging plant tissues and causing plant death cannot be ruled out. This may suggest either that the methanol metabolic pathway operating in plant roots would not be able to process methanol, as may occur in plant leaves, or that plant roots lack an effective pathway for processing methanol. Assuming that the pathway to metabolize methanol is functional in plant roots, an accumulation of methanol in the soil reaching toxic concentrations and causing plant death could be an explanation for

the methanol effect observed in the plants that were irrigated with the alcohol solution. It is difficult to ascertain whether we are observing a general toxicity response or the induction by methanol or one of its metabolites mediating these effects, or both. Human activities can also alter the natural balance between methanol levels in plants, the food chain, and the atmosphere. For example, if widespread agricultural use of methanol sprays gains favor (Fall and Benson 1996), or if the use of methanol fuels for vehicles increases dramatically (Reinchardt 1995), these technologies could have important indirect effects on methanol-dependent biological processes, probably in ways that cannot yet be predicted. Therefore, it is important to learn more about the plant system related to plant methanol biochemistry, physiology, and molecular biology.

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