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Changes in anatomy and terpene chemistry in roots of Douglas-fir seedlings following treatment with methyl jasmonate

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Summary Replicated trials were conducted on two full-sibling families of Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) seedlings. In response to the application of a 0.01% solution of methyl jasmonate (MeJA) to the soil of potted seedlings, numerous anatomical and chemical changes were observed in the roots, stem and foliage. These changes were, for the most part, similar for both sib groups. Methyl jasmonate induced traumatic resin duct formation in roots and stems. Chemical differences between MeJA-treated and control seedlings were mainly limited to the roots and stem, though some changes also occurred in the foliage. A total of 35 terpenoids were observed in the *P. menziesii* seedlings. In response to MeJA treatment, several of the 22 detected monoterpenoids (linalool, bornyl acetate, camphene, myrcene, α- and β-pinene, tricyclene and β-phellandrene) increased significantly in roots and stems, whereas (*E*)-β-ocimene decreased significantly in the foliage. Four of the five detected sesquiterpenoids (α-humulene, germacrene D, longifolene and (*E*)-caryophyllene) increased significantly following MeJA application, mainly in the root and stem. Four of the eight detected diterpenoids (abietate, levopimarate, palustrate and sandaracopimarate) increased in response to MeJA treatment, but only in root and stem tissue. This study provides the first description of the effects of MeJA applied to roots through the soil on the anatomy and terpene chemistry of a gymnosperm. This comprehensive inventory of terpenoids in *P. menziesii*, with and without MeJA treatment, may facilitate identification of terpenoid-related resistance traits. Potential practical applications of MeJA treatment of conifer roots as a pest management strategy are discussed.

Keywords: forest pathology, forest pest management, induced conifer defense, octadecanoid, oleoresin, Pseudotsuga menziesii, traumatic resin duct, tree biotechnology.

Introduction

The use of methyl jasmonate (MeJA) to induce defense responses in conifers, particularly spruce (*Picea* spp.), has been the focus of many recent studies (Kozlowski et al. 1999, Lapointe et al. 2001, Franceschi et al. 2002, Martin et al. 2002, 2003, Fäldt et al. 2003, Hudgins et al. 2004, Miller et al. 2005). Application of MeJA to stems causes several anatomical changes in spruce (Fransceschi et al. 2002, Martin et al. 2002, Miller et al. 2005) and other gymnosperms (Hudgins et al. 2004), particularly the formation of traumatic resin ducts in the newly developing xylem. Other noticeable anatomical changes following MeJA application to gymnosperm stems include resinosis, lignification and polyphenolic parenchyma cell activation (Hudgins et al. 2004).

The anatomical changes observed following the application of MeJA to stems and foliage of conifers are accompanied by changes in resin chemistry and, in particular, changes in the terpenoid components of the oleoresin. Following MeJA application, the concentrations of monoterpenoids and diterpenoids increase in stems of Norway spruce (*Picea abies* (L.) Karst.) and Sitka spruce (*Picea sitchensis* (Bong.) Carrière) (Martin et al. 2002, Miller et al. 2005), and there is a corresponding, but less pronounced, increase in monoterpenoid concentration in foliage (Martin et al. 2003). In addition, following MeJA treatment, increased amounts of some volatile monoterpenes and sesquiterpenes were emitted from Norway spruce and Sitka spruce foliage over a diurnal cycle with the highest release rates occurring during the light period (Martin et al. 2003, Miller et al. 2005).

The metabolic and molecular machinery associated with induced resin terpenoid formation is up-regulated following MeJA treatment (Martin et al. 2002, 2003, Miller et al. 2005). In Norway spruce, monoterpene and diterpene synthase activities increase in the xylem but not in the bark, paralleling the changes in resin chemistry (Martin et al. 2002). In addition, transcript levels of terpene synthase genes (Martin et al. 2004) increase in response to MeJA treatment (Fäldt et al. 2003, Miller et al. 2005). The anatomical, chemical, biochemical and molecular genetic changes seen in Norway spruce stems and foliage after MeJA treatment closely resemble those observed after mechanical or insect-associated damage (Alfaro 1995, Litvak and Monson 1998, Tomlin et al. 1998, 2000,

Alfaro et al. 2002, Byun MacKay et al. 2003, Miller et al. 2005), suggesting a role for MeJA or other octadecanoid compounds in the signaling pathway associated with induced defense responses in conifers.

Although MeJA-induced changes in stem and foliage tissues, particularly in spruce species, have been well studied, few studies have focused on the MeJA-induced anatomical and chemical responses in roots. In addition, relatively little is known about MeJA-induced defenses in Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) (Kaukinen et al. 1996, Hudgins et al. 2004), though it is of considerable economic and ecological importance in the Pacific Northwest and throughout the world in plantation forests. Hudgins et al. (2004) recently described induced anatomical defenses of Douglas-fir stem tissues as part of a larger survey of conifer responses to MeJA treatment. Douglas-fir roots are susceptible to a wide range of fungal and insect pests. Resistance traits in tree roots, though rarely studied, likely play a large role in protecting trees from such pests. We describe here changes in root anatomy and terpenoid chemistry following MeJA treatment and provide data that lay the groundwork for detailed investigations of the belowground defense mechanisms in Douglas-fir.

Materials and methods

Trees

Douglas-fir trees were grown from seed provided by the British Columbia Ministry of Forests, at the Canadian Forest Service's Pacific Forestry Centre in Victoria, BC, Canada. Two full-sibling families (Families 79 and 84) came from six-parent diallel crosses that had undergone extensive field-testing for growth performance on Vancouver Island and in southwestern British Columbia. Seeds were soaked for 48 h at room temperature, stratified at 2–5 °C for 28 days, and then sown in mid-March 2001 in Styroblock (Beaver Plastics, Edmonton, AB, Canada) containers (metric size 615 B) filled with a 2.5:1:1 (v/v) mix of peat:perlite:vermiculite amended with dolomite lime and Osmocote (N,P,K 18:6:12). Trees were transplanted to 4.5-l pots containing a 3:1:1 (v/v) mix of peat:vermiculite:sand (amended as for the Styroblock mix) during the week of November 27, 2001 and immediately moved to a greenhouse at the University of British Columbia.

Methyl jasmonate treatment and tissue collection

The MeJA treatment solution, which was prepared on the day of application, contained 0.01% (v/v) methyl jasmonate (Aldrich Chemical, Milwaukee, WI) and 0.1% (v/v) TWEEN 20 (Fisher Scientific, Nepean, ON, Canada) in deionized autoclaved water in capped 1-l glass bottles. The freshly prepared solution was stirred until no MeJA droplets were visible (about 1 h). The 0.1% (v/v) TWEEN 20 control solution was also prepared in deionized autoclaved water.

Seedlings selected for study were 14–15 months old and about 35 cm tall. Their pots were not watered for 2 days before treatment to ensure that all of the solution added to the pots was absorbed by the soil. Five trees in each of Familes 79 and 84 were designated as MeJA-treatment trees and five trees in each family were designated as controls. The MeJA treatment trees received 150 ml of MeJA-treatment solution, poured slowly into the soil close to the stem, on May 29 and again on June 12, 2002. Control trees received 150 ml of 0.1% (v/v) TWEEN 20 solution applied in a similar manner. Trees were not watered for 24 h after treatment application. Throughout the experiment (May 29 to July 3), the trees were maintained in a greenhouse in a natural photoperiod without an additional light source. Mean maximum temperature was 31.4 °C, mean minimum temperature was 19.0 °C and mean overall temperature was 22.5 °C during the experiment.

On July 3, 2002, tissue samples from each control and treatment tree were harvested and stored separately as follows. Foliage from the top 3–4 cm of the leader and lateral branches of each tree was flash frozen separately in liquid nitrogen for chemical analyses. The first 3 cm of stem tissue above the soil was discarded, the next 4 cm was flash frozen in liquid nitrogen for chemical analyses and the next 2 cm was fixed in FAA fixative solution (50% ethanol, 5% glacial acetic acid and 3.7% formaldehyde) for histological analysis. The first 2 cm of primary root below ground was discarded, the next 2–3 cm was flash frozen in liquid nitrogen for chemical analysis and the next 2–3 cm was fixed in FAA for histological analysis. All frozen tissues intended for resin terpenoid extraction were maintained in capped conical vials at –80 °C until extraction and all histological samples were maintained in capped glass tubes at room temperature in FAA fixative until sectioned.

Histological analyses and extraction of resin terpenoids

One week before histological analyses, stem and root sections stored in FAA were transferred twice (3 to 4 days each time) to 70% ethanol to partially soften the tissues for microtome sectioning. Softened stem and root tissues of all collected samples were sectioned to 60 µm, stained with safranin and placed on slides with glycerol. Sections were visualized with a Leica MS5 microscope (Richmond Hill, ON, Canada) and were digitally photographed with a Javelin SmartCam (Javelin Systems, Torrance, CA).

GC-FID and GC-MS analyses

Resin terpenoids were extracted from frozen root, stem and foliage tissues as described by Martin et al. (2002). Resin monoterpenoids and sesquiterpenoids were analyzed by gas chromatographic-flame ionization detection (GC-FID) with an Agilent 6890A Series GC system (Agilent Technologies, Palo Alto, CA) fitted with an Agilent 7683 Series autosampler running Agilent Chem Station Rev. A.09.01 software. The temperature program was: 40 °C held for 3 min, to 110 °C at 3 °C min^{-1}, to 180 °C at 10 °C min^{-1} to 250 °C at 15 °C min^{-1} and then held for 10 min. The column was DBWAX (J&W Scientific, Folsom, CA), 30 m × 0.25 mm ID (0.25-µm thickness); carrier gas was H_2 at 2 ml min^{-1} (constant flow); injection volumes were 1 µl and were split (5:1) with an injector temperature of 220 °C; FID temperature was 300 °C; H_2 flow was 40 ml min^{-1}; air flow was 450 ml min^{-1}; and N_2 flow was 50 ml min^{-1}.

The GC-FID conditions for analysis of resin diterpenoids were the same as those described for the analysis of monoterpenoids and sesquiterpenoids except for the following changes: the temperature program was 150 °C held for 1 min, to 182 °C at 0.5 °C min^{-1}, to 220 °C at 5 °C min^{-1}, to 300 °C at 20 °C min^{-1} and then held for 5 min. The column was an Agilent HP5 capillary, 30 m × 0.25 mm ID (0.25-μm thickness); carrier gas was H_2 at 2 ml min^{-1}; injection volumes were 1 μl and were pulsed split (50:1) with an injector temperature of 250 °C. Diterpenoids were methylated before analysis as described by Martin et al. (2002).

Gas chromatographic-mass spectroscopic (GC-MS) conditions for analyses of resin monoterpenoids and sesquiterpenoids were as follows: the GC-MS was an Agilent 6890A Series GC system fitted with an Agilent 7683 Series autosampler and a 5973N Mass Selective Detector (Agilent quadrupole MS) running Agilent Enhanced Chem Station version D.00.00.38 software; the temperature program was 40 °C held for 3 min, to 110 °C at 3 °C min^{-1}, to 180 °C at 5 °C min^{-1}, to 250 °C at 15 °C min^{-1} and then held for 20 min; column and injection conditions were the same as described for the GC-FID analyses of monoterpenoids and sesquiterpenoids; the MS, in electron ionization mode (70 eV), had a transfer line temperature of 280 °C, a solvent delay of 10 min, and a mass range setting of 40–350.

The GC-MS conditions for analyses of resin diterpenes were as follows: the GC-MS setup was the same as that described for GC-MS analyses of resin monoterpenoids and diterpenoids; the temperature program was 150 °C held for 1 min, to 182 °C at 0.5 °C min^{-1}, to 220 °C at 5 °C min^{-1}, to 300 °C at 20 °C min^{-1} and then held for 15 min; column and injection conditions were the same as described for the GC-FID analyses of diterpenoids except the injection was pulsed split (25:1); the MS, in electron ionization mode (70 eV), had a transfer line temperature of 280 °C, a solvent delay of 15 min, and a mass range setting of 40–400. Diterpenoids were methylated as described by Martin et al. (2002).

Compounds were identified by retention time matching with authentic standards and with Wiley275 (Wiley Mass Spectral Libraries, Rev. D.01.00, June 2000, purchased from Agilent Technologies) and HP1607 (purchased from Agilent Technologies) mass spectra libraries. Internal standards were isobutyl benzene for monoterpenoid and sesquiterpenoid analyses and methylated dichlorodehydroabietic acid for diterpenoid analyses.

Statistical analyses

Resin canal formations in root and stem sections of treated and control trees from both families were ranked separately by two workers and then compared. Resin canal rankings (RCR) were scaled as: 0 = no resin canal formations; 1 = scattered resin canals, no rings or partial rings; 2 = partial or incomplete ring with scattered canals; 3 = one complete ring; 4 = complete inner ring and incomplete outer ring with scattered formations; and 5 = two complete rings. The mean and standard error of the rankings for both treatments from both families ($n = 5$ for each family) were determined and compared separately for stem and root tissues by a one-tailed t test.

Differences in tissue concentrations of terpenoids were assessed separately for each terpenoid or putative terpenoid, in each tissue type and in each family ($n = 5$ in each case). Comparisons in each case were made with two-sample, two-tailed t tests assuming unequal variances (TTEST function, Microsoft Excel 2000).

Results

Histological analyses revealed substantial resin duct formation in roots and stems of Douglas-fir seedlings of both Families 79 and 84 (Figures 1 and 2) following application of MeJA to the soil. Resin duct formation was slightly more advanced in stems than in roots with mean RCR ≈ 4 for stems in both families compared with RCR ≈ 3 for roots (Figure 2). To ensure equal application of MeJA to each tree, trees were not watered for 2 days before treatment so that there was no runoff of MeJA solution. It was therefore necessary to control for the effect of mild drought by treating control trees in the same fashion, except without MeJA. Resin duct formation in stems and roots of both families was significantly greater in trees in the MeJA treatment than in trees in the TWEEN 20 control treatment (Figure 2), confirming that induced resin duct development was due to treatment with MeJA.

In both families, changes in the terpenoid chemistry of foliage (Table 1), stem (Table 2) and root (Table 3) following application of MeJA to the soil were similar, but not identical. In total, 22 monoterpenoids were identified in foliage, stem and root tissues. The amounts of several monoterpenoids were altered significantly in response to MeJA. Linalool increased significantly in foliage in Families 79 and 84 (2.3- and 10.5-fold, respectively), and (*E*)-β-ocimene decreased significantly in foliage in Family 79 (2.5-fold) (Table 1). Methyl jasmonate significantly increased the concentrations of several monoterpenoids in stem tissue in Families 79 and 84 including bornyl acetate (5.6- and 3.6-fold, respectively), camphene (4.0- and 3.7-fold, respectively), myrcene (1.6- and 2.6-fold, respectively), α-pinene (3.6- and 3.5-fold, respectively) and β-pinene (2.7- and 2.8-fold, respectively), whereas the treatment significantly increased tricyclene only in Family 79 (26-fold) and significantly increased β-phellandrene only in Family 84 (2.5-fold) (Table 2). In root tissue in Families 79 and 84, MeJA significantly increased tricyclene (5.2- and 17.7-fold, respectively), whereas the treatment significantly increased camphene (2.2-fold), β-phellandrene (2.0-fold) and β-pinene (2.3-fold) in Family 79 only and significantly increased α-pinene (4.3-fold) in Family 84 only (Table 3). Other monoterpenoids that showed substantial, but not statistically significant, changes in some tissues following MeJA application were 3-carene, citronellol, citronellyl acetate, limonene, γ-terpinene, terpineol, terpinolene and thujene.

In total, five sesquiterpenoids were identified in foliage, stem and root tissues. The concentrations of sesquiterpenoids in foliage were unaffected by the MeJA treatment except for

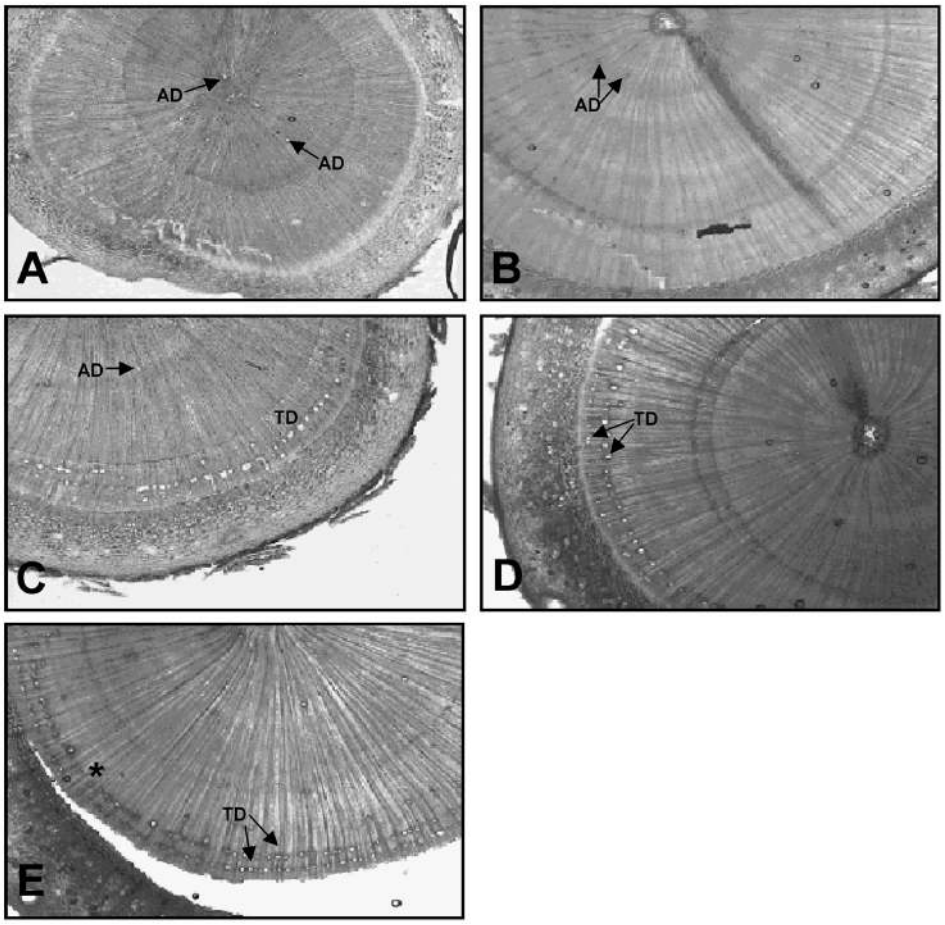

Figure 1. Induction of traumatic resin ducts in Douglas-fir in response to application of methyl jasmonate (MeJA) to the soil. (A) Root of control seedling and (B) stem of control seedling. Numerous axial resin ducts (AD) can be seen in the previous-year growth. (C and E) Root of seedling grown in MeJA-treated soil, and (D) stem of seedling grown in MeJA-treated soil. Note the ring of traumatic resin ducts (TD), running the circumference of the tree in the second-year growth. In (E), MeJA treatment produced two complete rings of TDs. Oleoresin can be seen filling some of the TDs (*; E).

α-humulene which increased significantly in Family 84 (4.5-fold) (Table 1). In stems, MeJA significantly increased germacrene D in Family 84 (10.3-fold) and longifolene in Families 79 and 84 (8.8- and 9.8-fold, respectively) (Table 2). In roots, (*E*)-caryophyllene increased significantly in Family 79 (14.5-fold) in response to MeJA treatment (Table 3). No other sesquiterpenoids showed substantial differences in concentration between MeJA treatment and control groups in any tissue.

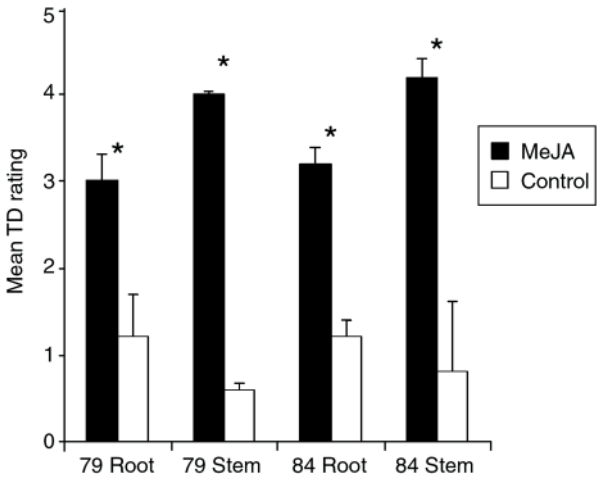

Figure 2. Traumatic resin duct (TD) formation in Douglas-fir after application of methyl jasmonate (MeJA) to the soil. Root and stem traumatic resin duct formation in the MeJA treatment and control groups in both families is based on the resin canal ranking system described in Methods. In all instances the treatment effects were significant (* = statistically significant difference ($P < 0.05$, *t* test, $n = 5$)).

In total, eight diterpenoids were identified in foliage, stem and root tissues. No diterpenoids were detected in the foliage of either family except for labdadien-8-ol, which was found in Family 84 foliage in both treatments (Table 1). In stems, MeJA significantly increased abietate in Families 79 and 84 (2.4- and 2.7-fold, respectively), and caused significant increases in levopimarate (11.9-fold), palustrate (25.3-fold) and sandaracopimarate (3.8-fold) in Family 84 (Table 2). In roots, MeJA application significantly increased abietate (1.8-fold) and levopimarate (2.1-fold) in Family 79 but not in Family 84 (Table 3). Although MeJA caused substantial changes in neoabietate in roots of both families and in dehydroabietate in stems of both families, neither effect was statistically significant.

Discussion

In long-lived coniferous trees, terpenoid-based defenses provide vital protection against a plethora of insect and fungal pests (Bohlmann and Croteau 1999, Phillips and Croteau 1999, Trapp and Croteau 2001, Huber et al. 2004). Methyl jasmonate has been shown to induce terpenoid defenses and re-

Table 1. Terpenoid concentration (mean ± 1 SE) in foliage of two families of Douglas-fir (*Pseudotsuga menziesii*) after soil application of methyl jasmonate (MeJA) solution compared to a control solution containing no MeJA. The MeJA treatment and control were compared for each terpenoid in each family with two-sample, two-tailed *t* tests assuming unequal variances (TTEST function, Microsoft Excel 2000, $n = 5$ in each case). Abbreviation: nd = not detected in any of the five replicates. Asterisks indicate statistically significant difference ($P = 0.05$) in at least one of the two families.

Compound	Family 79 ($\mu g\ g_{DM}^{-1}$)			Family 84 ($\mu g\ g_{DM}^{-1}$)		
	Control	MeJA	*P*	Control	MeJA	*P*
Monoterpenoids						
Bornyl acetate	5.5 ± 2.2	6.4 ± 1.9	0.75	7.1 ± 1.4	12.1 ± 4.0	0.29
Camphene	25.0 ± 4.9	21.2 ± 3.7	0.56	16.5 ± 1.4	24.1 ± 4.6	0.18
3-Carene	160.8 ± 34.7	160.2 ± 42.1	0.99	46.2 ± 9.0	80.2 ± 38.1	0.43
Citronellol	123.1 ± 23.1	169.8 ± 29.9	0.25	96.0 ± 23.6	169.4 ± 24.2	0.06
Citronellyl acetate	236.2 ± 38.7	288.4 ± 74.0	0.56	170.6 ± 20.7	225.7 ± 42.6	0.29
Cymene	nd	nd	–	nd	nd	–
Geranyl acetate	129.8 ± 23.5	202.4 ± 72.1	0.38	141.5 ± 38.5	243.7 ± 41.1	0.11
Limonene	48.9 ± 14.5	49.2 ± 15.4	0.99	76.7 ± 10.6	98.3 ± 20.2	0.38
Linalool*	9.0 ± 1.1	20.3 ± 3.8	0.04	6.8 ± 1.7	71.4 ± 22.5	0.05
Myrcene	115.5 ± 17.3	110.7 ± 35.4	0.91	162.5 ± 19.5	133.8 ± 10.2	0.24
(*E*)-β-Ocimene*	11.4 ± 2.0	4.5 ± 1.4	0.02	25.0 ± 3.5	28.7 ± 7.2	0.66
(*Z*)-β-Ocimene	30.8 ± 2.6	31.0 ± 3.5	0.96	39.0 ± 5.2	46.6 ± 4.1	0.29
β-Phellandrene	54.9 ± 11.2	45.3 ± 10.3	0.54	38.0 ± 3.8	60.8 ± 13.4	0.17
α-Pinene	551.8 ± 114.8	475.2 ± 100.2	0.63	425.7 ± 40.1	636.6 ± 124.5	0.17
β-Pinene	1921.1 ± 390.6	1687.4 ± 371.4	0.68	1450.2 ± 136.3	1968.9 ± 267.6	0.14
Sabinene	34.3 ± 9.6	30.5 ± 8.3	0.77	33.1 ± 8.9	64.4 ± 39.3	0.48
α-Selinene	29.0 ± 4.7	34.6 ± 5.8	0.47	23.4 ± 2.1	26.1 ± 3.6	0.55
γ-Terpinene	2.8 ± 1.2	4.2 ± 1.2	0.44	1.0 ± 0.5	1.8 ± 1.2	0.52
Terpineol	5.6 ± 1.9	11.0 ± 1.9	0.08	11.5 ± 5.5	37.9 ± 12.3	0.10
Terpinolene	12.6 ± 3.5	16.0 ± 3.6	0.51	8.8 ± 1.9	10.9 ± 5.1	0.71
Thujene	0.0 ± 0.0	0.2 ± 0.2	0.37	0.0 ± 0.0	0.4 ± 0.4	0.37
Tricyclene	4.1 ± 1.4	2.3 ± 1.0	0.34	2.0 ± 0.8	2.3 ± 1.0	0.80
Sesquiterpenoids						
(*E*)-Caryophyllene	41.8 ± 8.7	43.6 ± 7.2	0.88	37.2 ± 15.3	49.9 ± 19.8	0.63
α-Cedrene	nd	nd	–	nd	nd	–
Germacrene D	143.2 ± 28.0	189.3 ± 42.8	0.40	215.8 ± 30.2	186.2 ± 14.0	0.41
α-Humulene*	4.3 ± 0.5	4.3 ± 1.5	0.99	0.6 ± 0.3	2.7 ± 0.6	0.03
Longifolene	nd	nd	–	nd	nd	–
Diterpenoids						
Abietate	nd	nd	–	nd	nd	–
Dehydroabietate	nd	nd	–	nd	nd	–
Isopimarate	nd	nd	–	nd	nd	–
Labdadien-8-ol	nd	nd	–	6.1 ± 6.1	23.2 ± 17.7	0.40
Levopimarate	nd	nd	–	nd	nd	–
Neoabietate	nd	nd	–	nd	nd	–
Palustrate	nd	nd	–	nd	nd	–
Sandaracopimarate	nd	nd	–	nd	nd	–

lated anatomical changes in Norway spruce (Martin et al. 2002, 2003, Franceschi et al. 2002) and Sitka spruce (Miller et al. 2005) and anatomical changes in a suite of other conifers (Hudgins et al. 2004), but, to the best of our knowledge, this is the first report of the chemical effects of MeJA in Douglas-fir. We investigated the chemical and anatomical responses of Douglas-fir seedlings to MeJA applied to roots by soil irrigation. The consistency of the anatomical and chemical changes in root and stem tissues between two families with different genetic backgrounds indicates the general characteristics of MeJA-induced responses in Douglas-fir.

Application of MeJA to soil caused detectable effects in Douglas-fir seedings, but they were limited mainly to the root and stem at 21 days after the second and final MeJA application. Although some foliage effects were seen following application of MeJA to soil, in particular an increase in linalool in both families and a decrease in (*E*)-β-ocimene in Family 84, there were no other statistically significant changes in terpenoid concentrations in foliage. Similar results were obtained by Martin et al. (2003), who demonstrated that topical application of MeJA to foliage had little long-term effect on foliage terpenoid concentration in Norway spruce, although there was a

Table 2. Terpenoid concentration (mean ± 1 SE) in stems of two families of Douglas-fir (*Pseudotsuga menziesii*) after soil application of methyl jasmonate (MeJA) solution compared to a control solution containing no MeJA. The MeJA treatment and control were compared for each terpenoid in each family with two-sample, two-tailed *t* tests assuming unequal variances (TTEST function, Microsoft Excel 2000, $n = 5$ in each case). Abbreviation: nd = not detected in any of the five replicates. Asterisks indicate statistically significant difference ($P = 0.05$) in at least one of the two families. Actual *P* values were slightly higher than 0.05 in two instances, indicated by ** beside Family 79, dehydroabietate, $P = 0.0522$ and Family 79, levopimarate, $P = 0.0524$.

Compound	Family 79 (μg g_{DM}^{-1})			Family 84 (μg g_{DM}^{-1})		
	Control	MeJA	*P*	Control	MeJA	*P*
Monoterpenoids						
Bornyl acetate*	1.6 ± 0.5	9.0 ± 2.6	0.05	2.9 ± 0.5	10.3 ± 1.5	0.01
Camphene*	7.1 ± 1.0	28.2 ± 4.9	0.01	5.9 ± 0.9	21.6 ± 3.5	0.01
3-Carene	273.3 ± 58.4	178.1 ± 8.9	0.18	182.1 ± 33.1	374.7 ± 87.5	0.09
Citronellol	13.0 ± 2.7	6.5 ± 1.8	0.08	13.0 ± 5.3	13.4 ± 3.9	0.96
Citronellyl acetate	46.9 ± 10.2	22.5 ± 3.3	0.07	45.8 ± 14.1	75.5 ± 13.3	0.16
Cymene	0.5 ± 0.3	1.5 ± 0.9	0.33	0.7 ± 0.7	2.1 ± 0.9	0.27
Geranyl acetate	6.4 ± 1.6	4.2 ± 1.4	0.33	8.3 ± 3.0	12.3 ± 4.4	0.48
Limonene	19.3 ± 6.8	44.7 ± 11.2	0.10	50.9 ± 19.2	118.3 ± 29.5	0.10
Linalool	0.4 ± 0.4	2.0 ± 1.5	0.36	0.8 ± 0.4	2.3 ± 1.2	0.27
Myrcene*	29.4 ± 5.2	46.1 ± 3.0	0.03	31.5 ± 3.5	81.6 ± 11.1	0.01
(*E*)-β-Ocimene	0.4 ± 0.4	0.2 ± 0.1	0.72	11.4 ± 4.2	9.0 ± 4.0	0.69
(*Z*)-β-Ocimene	0.0 ± 0.0	0.3 ± 0.2	0.24	0.0 ± 0.0	0.1 ± 0.1	0.37
β-Phellandrene*	19.3 ± 2.9	32.1 ± 5.8	0.10	14.1 ± 1.1	35.1 ± 4.8	0.01
α-Pinene*	498.7 ± 90.6	1779.0 ± 253.9	0.01	404.6 ± 73.9	1397.6 ± 237.5	0.01
β-Pinene*	106.8 ± 19.4	288.8 ± 33.3	< 0.01	99.8 ± 13.0	274.6 ± 46.8	0.02
Sabinene	88.1 ± 20.4	84.7 ± 7.1	0.88	105.2 ± 15.3	244.1 ± 80.4	0.16
α-Selinene	0.0 ± 0.0	0.0 ± 0.0	0.37	nd	nd	–
γ-Terpinene	6.2 ± 1.3	7.3 ± 2.3	0.69	4.9 ± 0.4	10.9 ± 2.3	0.06
Terpineol	2.7 ± 1.3	4.3 ± 1.0	0.34	0.7 ± 0.2	8.8 ± 3.9	0.10
Terpinolene	53.6 ± 14.4	73.0 ± 20.0	0.46	36.0 ± 2.6	131.6 ± 37.9	0.07
Thujene	1.1 ± 0.4	2.1 ± 0.4	0.14	1.2 ± 0.4	3.6 ± 1.1	0.08
Tricyclene*	0.1 ± 0.1	2.6 ± 0.4	< 0.01	0.0 ± 0.0	1.5 ± 0.6	0.08
Sesquiterpenoids						
(*E*)-Caryophyllene	0.0 ± 0.0	0.8 ± 0.5	0.17	6.7 ± 1.6	4.9 ± 2.5	0.58
α-Cedrene	0.0 ± 0.0	2.0 ± 1.2	0.18	nd	nd	–
Germacrene D*	4.0 ± 0.7	3.8 ± 0.4	0.80	0.6 ± 0.3	6.2 ± 1.6	0.03
α-Humulene	1.7 ± 0.3	3.5 ± 0.8	0.08	0.9 ± 0.4	1.8 ± 0.8	0.34
Longifolene*	0.5 ± 0.5	4.4 ± 1.4	0.04	0.8 ± 0.8	7.8 ± 2.3	0.04
Diterpenoids						
Abietate*	758.0 ± 110.8	1832.8 ± 333.5	0.03	637.7 ± 63.3	1731.1 ± 121.5	< 0.01
Dehydroabietate**	316.7 ± 39.5	741.4 ± 157.2	0.05	472.5 ± 140.8	931.0 ± 186.7	0.09
Isopimarate	244.2 ± 46.0	319.1 ± 92.7	0.50	296.7 ± 24.6	340.1 ± 26.9	0.27
Labdadien-8-ol	554.5 ± 144.2	750.7 ± 148.8	0.37	370.5 ± 134.6	1595.8 ± 683.4	0.15
Levopimarate**	399.7 ± 102.9	1704.0 ± 482.2	0.05	96.5 ± 46.7	1145.8 ± 260.1	0.01
Neoabietate	197.0 ± 62.4	315.0 ± 58.4	0.20	0.0 ± 0.0	159.8 ± 71.2	0.09
Palustrate*	94.9 ± 29.5	582.3 ± 223.6	0.09	11.1 ± 11.1	281.1 ± 70.3	0.02
Sandaracopimarate*	69.9 ± 18.0	184.1 ± 53.6	0.10	48.3 ± 16.5	183.0 ± 14.2	< 0.01

short-term increase in monoterpene and sesquiterpene concentrations in foliage (for ~15 days) in Norway spruce and the volatile release of monoterpenoids and sesquiterpenoids increased on a diurnal cycle over at least 6 days immediately following treatment. The oxygenated monoterpenoid, (−)-linalool, was a major component of the emissions of MeJA-treated Norway spruce (Martin et al. 2003) and Sitka spruce (Miller et al. 2005). It was hypothesized that the volatile terpenoids were released as they were synthesized and did not accumulate in the foliage (Martin et al. 2003, Miller et al. 2005). We are currently testing this hypothesis in Douglas-fir. An increased concentration of linalool in foliage suggests some form of systemic signaling in Douglas-fir seedlings treated with MeJA by soil irrigation. Alternately, foliage could respond to low concentrations of MeJA evaporating from the treated soil.

Insect attack, wounding and MeJA treatment of stems and foliage induces increases in gene transcripts for terpene synthases in Norway spruce and in Sitka spruce (Byun McKay et al. 2003, Fäldt et al. 2003, Miller et al. 2005). Similarly, stem and foliage application of MeJA in Norway spruce elevates terpene synthase activity and the activity of geranylgeranyl diphosphate synthase in the wood, and subsequently, the con-

Table 3. Terpenoid concentration (mean ± 1 SE) in roots of two families of Douglas-fir, *Pseudotsuga menziesii*, after soil application of methyl jasmonate (MeJA) solution compared to a control solution containing no MeJA. The MeJA treatment and control were compared for each terpenoid in each family with two-sample, two-tailed *t* tests assuming unequal variances (TTEST function, Microsoft Excel 2000, $n = 5$ in each case). Abbreviation: nd = not detected in any of the five replicates. Asterisks indicate statistically significant difference ($P = 0.05$) in at least one of the two families. In one instance, the actual *P* value was slightly higher than 0.05, indicated by ** beside Family 84, camphene, $P = 0.0504$.

Compound	Family 79 ($\mu g\ g_{DM}^{-1}$)			Family 84 ($\mu g\ g_{DM}^{-1}$)		
	Control	MeJA	*P*	Control	MeJA	*P*
Monoterpenoids						
Bornyl acetate	6.9 ± 2.0	13.5 ± 2.6	0.09	5.8 ± 1.6	31.2 ± 10.1	0.06
Camphene**	12.8 ± 3.0	28.2 ± 3.1	0.01	8.9 ± 2.8	52.2 ± 15.8	0.05
3-Carene	128.9 ± 52.8	226.9 ± 49.2	0.21	104.9 ± 8.7	418.1 ± 118.3	0.06
Citronellol	nd	nd	–	0.0 ± 0.0	0.2 ± 0.2	0.37
Citronellyl acetate	0.2 ± 0.1	0.6 ± 0.2	0.18	1.4 ± 0.4	3.0 ± 0.6	0.07
Cymene	0.7 ± 0.7	0.9 ± 0.6	0.82	0.6 ± 0.6	8.8 ± 8.2	0.38
Geranyl acetate	nd	nd	–	0.1 ± 0.1	0.5 ± 0.3	0.35
Limonene	34.7 ± 17.8	68.5 ± 24.6	0.30	71.4 ± 33.1	319.7 ± 175.2	0.23
Linalool	0.0 ± 0.0	0.2 ± 0.2	0.37	0.7 ± 0.4	0.0 ± 0.0	0.12
Myrcene	29.2 ± 9.1	61.1 ± 12.8	0.08	34.0 ± 9.1	157.6 ± 60.7	0.11
(*E*)-β-Ocimene	nd	nd	–	0.0 ± 0.0	0.2 ± 0.2	0.37
(*Z*)-β-Ocimene	nd	nd	–	nd	nd	–
β-Phellandrene*	15.9 ± 4.3	31.2 ± 4.1	0.03	12.2 ± 1.4	56.6 ± 17.5	0.06
α-Pinene*	926.1 ± 246.2	1625.4 ± 215.4	0.07	650.7 ± 216.1	2794.4 ± 765.0	0.05
β-Pinene*	124.8 ± 32.7	289.5 ± 21.3	< 0.01	79.1 ± 23.2	512.9 ± 235.4	0.14
Sabinene	60.0 ± 37.0	59.8 ± 12.7	1.00	78.6 ± 26.2	186.3 ± 107.8	0.38
α-Selinene	nd	nd	–	0.0 ± 0.0	0.2 ± 0.2	0.37
γ-Terpinene	2.7 ± 1.5	5.3 ± 1.1	0.20	3.2 ± 0.6	13.6 ± 6.2	0.17
Terpineol	0.1 ± 0.1	2.2 ± 1.0	0.11	0.9 ± 0.9	7.6 ± 5.8	0.31
Terpinolene	43.7 ± 20.5	69.2 ± 14.8	0.35	48.8 ± 9.6	165.4 ± 70.1	0.17
Thujene	0.7 ± 0.7	0.8 ± 0.3	0.82	1.0 ± 0.5	4.6 ± 3.0	0.31
Tricyclene*	0.6 ± 0.4	3.1 ± 0.5	< 0.01	0.3 ± 0.3	5.3 ± 1.5	0.03
Sesquiterpenoids						
(*E*)-caryophyllene*	0.2 ± 0.2	2.9 ± 0.9	0.04	4.3 ± 2.2	5.9 ± 2.7	0.66
α-Cedrene	nd	nd	–	nd	nd	–
Germacrene D	nd	nd	–	0.0 ± 0.0	1.2 ± 0.7	0.17
α-Humulene	2.7 ± 1.4	0.0 ± 0.0	0.13	nd	nd	–
Longifolene	0.7 ± 0.7	7.7 ± 3.7	0.14	0.8 ± 0.8	22.5 ± 10.6	0.11
Diterpenoids						
Abietate*	746.0 ± 195.0	1359.4 ± 108.8	0.03	695.7 ± 339.8	2340.9 ± 879.6	0.14
Dehydroabietate	213.1 ± 132.6	364.9 ± 173.2	0.51	400.7 ± 290.6	972.7 ± 681.7	0.47
Isopimarate	405.7 ± 153.0	391.6 ± 87.4	0.94	524.9 ± 129.6	776.8 ± 320.2	0.50
Labdadien-8-ol	168.1 ± 86.4	165.1 ± 68.1	0.98	3.8 ± 3.8	185.3 ± 185.3	0.38
Levopimarate*	1302.3 ± 307.7	2777.2 ± 320.6	0.01	1455.5 ± 562.9	5253.8 ± 1549.9	0.07
Neoabietate	430.2 ± 112.3	719.9 ± 22.5	0.06	278.6 ± 75.8	942.6 ± 268.7	0.07
Palustrate	783.0 ± 190.3	1260.3 ± 87.8	0.07	835.2 ± 273.6	2542.6 ± 781.6	0.09
Sandaracopimarate	90.1 ± 35.8	149.7 ± 13.6	0.18	109.6 ± 48.6	323.7 ± 124.1	0.17

centrations of terpenoids in the wood and to some extent in the bark of the stem are also elevated (Martin et al. 2002). Although we did not separate bark from wood in our analyses, we found similar increases in terpenoids in entire stems of Douglas-fir seedlings. In stems of Family 79, the concentrations of six monoterpenoids, one sesquiterpenoid and one diterpenoid showed statistically significant ($P = 0.05$) increases in Douglas-fir seedlings treated with MeJA by soil irrigation compared with control trees. Similarly, in stems of Family 84, the concentrations of five monoterpenoids, two diterpenoids and four sesquiterpenoids increased in response to the MeJA treatment.

In roots of Family 79, the concentrations of four monoterpenoids, one sesquiterpenoid and two diterpenoids increased significantly in response to soil application of MeJA for five independent seedling experiments. In roots of Family 84, MeJA caused increased concentrations of two monoterpenoids, but had no effect on the concentrations of sesquiterpenoids and diterpenoids. However, almost all other monoterpenes and diterpenes detected also increased substantially in roots of treated seedlings in both families (Table 3), suggesting some major changes in the activities of the family of terpene synthases in roots of Douglas-fir seedlings. The finding that not all changes were significant is not surprising because the

seedlings were half-sibs with some inherent genetic diversity among the five replicates analyzed for each family. It has previously been shown that terpene composition varies substantially among seedlings of non-clonal trees of grand fir (*Abies grandis* (D. Don ex Lamb.) Lindl.; Katoh and Croteau 1998). Such variability is at least partially a result of multigenic control of complex mixtures of terpenoid profiles by large families of terpenoid synthase genes (Martin et al. 2004). Several functionally diverse terpene synthase genes have recently been identified in Douglas-fir (D.P.W. Huber, R.N. Philippe, K.-A. Goddard and J. Bohlmann, unpublished results). In conclusion, we have shown that soil application of MeJA to Douglas-fir seedlings strongly affects the terpenoid chemistry mainly of the root and stem tissues. Effects were noted both in the qualitative composition of terpenoid mixtures and in the total amount of terpenoids in the root and stem, and to some degree, also in the foliage.

Various anatomical changes, including the formation of traumatic resin ducts (TDs), occur in the developing xylem of trees following insect attack (Alfaro 1995, Alfaro et al. 2002, Byun McKay et al. 2003), fungal inoculation (Franceschi et al. 2000) or mechanical wounding (Tomlin et al. 1998, Nagy et al. 2000, Byun McKay et al. 2003, Hudgins et al. 2003). The resin content of the induced TDs differs from constitutive resin (Tomlin et al. 2000). It is thought that the development of TDs allows trees to move induced resin to the site of damage as a rapid and targeted defense against herbivores or invading pathogens (Alfaro et al. 2002) and acts to increase induced resin biosynthesis (Martin et al. 2002). The development of TDs in the developing xylem of spruce stems (Franceschi et al. 2002, Martin et al. 2002) and in the stems of several other gymnosperms including Douglas-fir, *A. grandis*, *Cedrus libani* A. Rich., *Tsuga heterophylla* (Raf.) Sarg., *Metasequoia glyptostroboides* H. H. Hu & Cheng., *Sequoia sempervirens* (D. Don) Endl. and *Sequoiadendron giganteum* (Lindl.) Buchh. (Hudgins et al. 2004) also occurs after topical application of MeJA to aerial parts, suggesting a role for octadecanoids in the signaling pathway for this anatomical change. Our result in stems of Douglas-fir parallels the results in other tree species following MeJA treatment, and we now provide evidence for a similar effect in the roots of a conifer. The TDs in the root and stem xylem of Douglas-fir following MeJA application to the soil were well developed by 5 weeks after treatment (Figures 1 and 2). Although we detected no resin exudation on stems in response to MeJA soil treatment in this study, in other work, MeJA sprayed on Douglas-fir trees caused swelling of resin blisters in stem bark tissues (data not shown).

Many organisms attack the roots and lower stem of Douglas-fir seedlings in nurseries in the Pacific Northwest, including insects, fungi and fungus-like stramenopiles, and nematodes. In the forests of the Pacific Northwest, Douglas-fir is also susceptible to extensive damage by several root pathogens including *Phellinus weirii* and *Armillaria ostoyae* (Allen et al. 1996). It has previously been shown that MeJA treatment can protect Norway spruce seedlings against *Pythium ultimum* (Kozlowski et al. 1999). Our finding that soil application of MeJA to Douglas-fir roots induces chemical and anatomical defense responses raises the possibility of treating seedlings with MeJA added to irrigation water in nurseries for protection against pests and pathogens. We are currently testing if pretreatment of seedlings with MeJA in a nursery before outplanting provides protection against root pathogens like *P. weirii*.

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