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VARIABLE CHEMISTRY AND HERBIVORY OF PONDEROSA PINE CONES

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We measured the terpenoid chemistry, cone insect distribution, and the relationship between these two parameters in the seed cones of ponderosa pine. Analyses of mono-, sesqui-, and diterpenes from four separate sites revealed high amounts of terpenoid diversity and variation. The majority of this variation occurred among trees within sites, but differences were also seen among sites and among cone clusters from individual trees. Cone insect distributions differed substantially in both time and space, with significant differences seen between two points in time and between five sites. Negative correlations existed between levels of cone insect herbivory and both a monoterpene and a diterpene factor at one site, indicating that these herbivores may be one reason that ponderosa pine maintains the high levels of chemical variation observed.

Keywords: Pinus ponderosa, cone insects, monoterpenes, diterpenes, chemical variation, herbivore variation.

Online enhancement: appendix table.

Introduction

Plants contain great variation and redundancy in the amounts and types of secondary compounds they produce. This variation extends from the classes of compounds produced among different plant families (Rosenthal and Berenbaum 1991; Romeo et al. 1996), among individuals and populations of single species (Thompson 2002), and even within individuals (Litvak and Monson 1998; Latta et al. 2000). Explanations for this diversity include the idea that producing multiple chemicals increases the chances of having compounds active against herbivores (Firn and Jones 2003), that chemical variation makes plants unpredictable to herbivores (Shelton 2000, 2004), that chemical diversity increases defense effectiveness due to the synergism possible in mixtures (Langenheim 1994; Cates 1996), and that the induction of plant secondary compounds in response to herbivores increases variation and diversity (Karban and Baldwin 1997). In addition to these biotic factors, plant chemistry may also vary as a result of environmental influences, such as water, nutrients, or light (Muzika et al. 1989; Kainulainen et al. 1992; Johnson et al. 1997).

In their defensive role, plant secondary compounds must deter a great variety of pathogens and herbivores (Linhart 1991). These herbivores vary both temporally and spatially (Denno and McClure 1983) and, depending on whether they are generalists or specialists, often differ in their rates and abilities to metabolize or detoxify plant defensive compounds (Schuler 1996; Boyle et al. 1999). In addition, specialist herbivores have evolved to tolerate host chemicals and even use them to find their hosts (Macias-Samano et al. 1998; Luik et al. 1999) or to protect themselves (Nogueira-de-Sa and Trigo 2005; Weiss 2006).

Terpenoids represent the primary chemical defense in conifers, occurring as a mixture of volatile monoterpenes (C_{10}) and sesquiterpenes (C_{15}), which solvate higher molecular weight diterpene resin acids (C_{20}), collectively known as oleoresin (Himejima et al. 1992; Trapp and Croteau 2001). Oleoresin acts as both a chemical defense, because of the activity of single constituents (Elliger et al. 1976; Marby and Gill 1979; Cates and Alexander 1982; Larsson et al. 1986; Kopper et al. 2005; Keeling and Bohlmann 2006 and references therein), and a physical defense, because of its ability to expel some herbivores with resin pressure and to crystallize and protect wound sites (Cates and Alexander 1982; Tomlin et al. 2000; Trapp and Croteau 2001). The ratio of lower molecular weight monoterpenes (and some sesquiterpenes) to diterpenes determines the viscosity of oleoresin (Hanover 1975; Tomlin et al. 2000). This physical property dictates how resin flows within a plant and, as a result, the ability of an internal parasite to move through it or the capacity of an external herbivore to eat tissue that contains it.

In ponderosa pine, numerous studies have documented variation in terpenoid chemistry across geographic regions (Smith 1977, 2000; von Rudloff and Lapp 1992), within populations (Zavarin and Cobb 1970; Latta et al. 2003; Thoss and Byers 2006), with season and needle age (Zavarin et al. 1971), and both within and among different tissues in individual trees (Litvak and Monson 1998; Latta et al. 2000). Most of these studies focused on the chemical variation of monoterpenes, and only a few researchers examined diterpene resin acid diversity (Joye et al. 1969; Fujii and Zinkel 1984; Zinkel and Magee 1991; Wagner et al. 1997).

Over its entire range, many pathogens and herbivores attack ponderosa pine, including pathogenic fungi brought by bark beetles (*Dendroctonus* spp. and *Ips* spp.); parasitic

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plants, such as dwarf mistletoes (*Arceuthobium* ssp.); mammals, such as the Abert's squirrel (*Sciurus abertii*) and porcupines (*Erethizon dorsatum*); and more than 200 species of insects (Furniss and Carolin 1980; Snyder 1992; Snyder et al. 1996; Snyder and Linhart 1997; Thoss and Byers 2006). One group of specialized insects feeds on maturing seed cones and can have a significant effect on the number of viable seeds produced (Hedlin et al. 1981; Cibrián-Tovar et al. 1986; Pasek and Dix 1988; Turgeon et al. 1994). After particularly heavy years of infestation, high cone and seed mortality can curtail recruitment of seedlings, potentially affecting the fitness of individual trees and stand dynamics (Kinzer et al. 1970; Bodenham and Stevens 1981; Schmid et al. 1984, 1986*a*, 1986*b*; Whitham and Mopper 1985; Pasek and Dix 1988; Blake et al. 1989).

While both the chemical and physical properties of ponderosa oleoresin deter pathogens (Himejima et al. 1992), parasites (Snyder et al. 1996), and a variety of herbivores (Cates and Alexander 1982; Snyder 1992; Snyder and Linhart 1994, 1997), few studies have looked at the relationship between terpenes and cone herbivores (Latta and Linhart 1997). Furthermore, only limited work has documented monoterpene variation in ponderosa pine cones (Latta et al. 2000) or terpenes in the cones of any conifer species (Yano and Furuno 1994; Kurose and Yatagai 2005; Otto et al. 2007; Sultan et al. 2008; Ucar and Ucar 2008).

In this study, we asked three questions. (1) Do the seed cones of ponderosa pine show geographic variation in their terpenoid chemistry? (2) Does the species composition of cone insects that attack ponderosa pine vary over time and among geographic locations? (3) Does a relationship exist between cone chemistry and levels of cone insect damage?

Material and Methods

Study Organisms

Ponderosa pine (*Pinus ponderosa* Douglas ex Laws) represents one of the widest-ranging tree species in North America, occurring from southern Canada into Mexico and from the Plains states of Nebraska and Oklahoma to the Pacific Coast from sea level to 3000 m. The development of seed cones takes place during two successive growing seasons (Pasek and Dix 1988). Wind pollination of female flower buds happens during spring of the first year, and fertilization takes place early in the second summer, after which cones expand to full size. Later in the same year, usually in September or early October, scales of the mature cones open, releasing the winged seeds.

At least 60 species of insects feed on and in the second-year green cones of ponderosa pine, with many occurring throughout the range of the tree but with varying local distributions (Furniss and Carolin 1980; Bodenham and Stevens 1981; Hedlin et al. 1981; Cibrián-Tovar et al. 1986; Pasek and Dix 1988; Furniss 1997). In the Colorado Front Range, the most prevalent cone-feeding insects include the cone beetle *Conophthorus ponderosae* Hopkins (Coleoptera: Scolytidae), the cone weevil *Conotrachelus neomexicanus* Fall (Coleoptera: Curculionidae), and the cone moths *Dioryctria* spp. (Lepidoptera: Pyralidae) and *Eucosma* spp. (Lepidoptera: Tortricidae; Bodenham and Stevens 1981). Adults of these species oviposit either on (cone weevil and moths) or in (cone beetle) green second-year cones in spring and early summer, and their larvae mine the interior, indiscriminately devouring scales and seeds (Furniss and Carolin 1980; Hedlin et al. 1981; Cibrián-Tovar et al. 1986). Depending on the density or species, these insects will destroy some or all of the seeds in a cone, often trapping remaining viable seeds in damaged cones that never open (Kinzer et al. 1970; Bodenham et al. 1976; Schmid et al. 1986*a*, 1986*b*; Pasek and Dix 1988; Blake et al. 1989). Infested cones quickly die, turn reddish to dark brown, and appear stunted or deformed.

Field Sites

We chose field sites located in the southern half of Boulder County, Colorado, covering a roughly linear east to west elevational transect of ~28 km, beginning 7.5 km southeast of the University of Colorado at Boulder on the plains (Marshall Mesa site, 39°57.089′N, 105°13.467′W; 1730 m) and continuing into the foothills of the Rockies to 3 km southwest of Ward, Colorado (Niwot site, 40°02.911′N, 105°31.535′W; 2950 m). Ponderosa pine represented the dominant tree species at the Marshall Mesa, Boulder Canyon (40°00.786′N, 105°18.176′W; 1700 m), Betasso Park (40°00.982′N, 105°20.820′W; 2000 m), and Bald Mountain (40°02.836′N, 105° 20.500′W; 2120 m) sites. Besides ponderosa pine, the Sugarloaf Mountain site (40°01.394′N, 105°25.909′W; 2600 m) also contained Douglas fir (*Pseudotsuga menziesii*) and limber pine (*Pinus flexilis*), and the Niwot site also had subalpine fir (*Abies lasiocarpa*), Engelmann spruce (*Picea engelmannii*), and limber pine. All sites had a similar slope and aspect with southern exposures.

Terpenoid Analysis

We measured the concentrations of 22 different terpenoids known to occur in ponderosa pine oleoresin (Joye et al. 1969; Smith et al. 1969; Zinkel and Magee 1991; von Rudloff and Lapp 1992; Wagner et al. 1997; Smith 2000) from seed cones. Monoterpenes assayed included α-pinene, camphene, β-pinene, δ-3-carene, myrcene, limonene, β-phellandrene, γ-terpinene, and terpinolene. We also measured the levels of a single sesquiterpene, longifolene, and nine diterpene resin acids, including levopimaric, palustric, isopimaric, abietic, dehydroxyabietic, neoabietic, imbricatocolic, succinylisocupressic, and acetylisocupressic acids. In addition, the diterpene analyses included three unknown compounds, which appeared consistently in all of the diterpene samples.

We collected green second-year seed cones with no apparent herbivore damage for terpenoid analysis between May 17 and June 15, 1999, at Marshall Mesa, Boulder Canyon, Betasso Park, and Sugarloaf Mountain. We chose these sites since they most represented the typical ponderosa habitat in our region. At each site, we haphazardly chose 10 cone-bearing trees and arbitrarily selected two cones each from two clusters on separate branches, with no regard to a particular side of the tree, for a total of 40 cones at each of the four sites ($N = 160$). We placed each cone in a separate polyethylene bag, double wrapped sets from each tree, and put them in a −60°C freezer within 4 h of collection until analysis.

We removed cones from the −60°C freezer as needed, bisected them, quickly ground one half in a coffee grinder, and further powdered the ground tissue with liquid nitrogen in a chilled mortar and pestle to minimize monoterpene loss. We weighed the second half of each cone and later dried them at 60°C to a constant weight to obtain cone dry weight. Monoterpenes and longifolene analysis included weighing ~0.6 g of the frozen cone powder into a small glass vial and adding 4.00 mL of an internal standard solution, which consisted of 0.1 μL/mL fenchone in *n*-pentane, a terpene that does not occur in ponderosa pine (Latta et al. 2000). We immediately sealed the vials with polytetrafluoroethylene-lined caps, mixed them with a vortex mixer, and left them to soak for 7 d at ambient temperature in the dark. After the 7-d soaking period, we withdrew a portion of the solution from each monoterpene/longifolene sample for direct analysis. In addition, to test whether the 7-d extraction period was appropriate, we injected aliquots from 10 of the monoterpene/longifolene samples again after 14 d and compared the results with those from the 7-d extractions.

We injected 2 μL of each monoterpene/longifolene sample on a Hewlett Packard 5890 gas chromatograph (GC) equipped with a flame ionization detector (FID) and fitted with a DB-Wax glass capillary column (15 m × 0.25 mm i.d., 0.25 μm film thickness; J&W Scientific), using helium as the carrier gas at a flow rate of 1.3 mL/min with a split flow ratio of 70 : 1. We set the injector temperature at 260°C and the detector at 250°C. The oven profile consisted of an isothermal hold at 50°C for 4 min, followed by a ramp of 8°C/min to 68°C, then a second ramp of 25°C/min to 240°C. We calculated concentrations of the monoterpenes and longifolene by comparison with injections of known amounts of pure standards, using fenchone as an internal standard (all standards from Sigma except β-phellandrene, which was from Glidco Organics [Jacksonville, FL]).

We carried out additional mono- and sesquiterpene identification analyses with an Agilent 6890N GC coupled with an Agilent 5975C inert mass selective detector with an ion source of 70.0 eV at 230°C, also using helium as the carrier gas at a flow rate of 1.0 mL/min and an injector temperature of 260°C. These analyses used an EC-Wax glass capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Alltech) with oven conditions that included an initial oven temperature of 40°C followed by an immediate ramp of 3°C/min to 200°C. We injected 1 μL of selected samples in the splitless mode and identified terpenes using retention times and mass spectra of pure standards, the NIST 2005 mass spectral library, and the study by Adams (2007).

We also used a continuous series of *n*-alkanes (C_8–C_{24}; Sigma-Aldrich) to calculate mono- and sesquiterpene linear retention indexes on the same 15-m DB-Wax and 30-m EC-Wax columns used in the above analyses and with an HP-5MS capillary column (30 m × 0.25 mm i.d., 0.25 μm film thickness; Agilent Technologies) installed on the GC/MS. All GC conditions remained the same as above, except for the oven profile, which for all retention index runs consisted of an initial temperature of 40°C followed by an immediate ramp of 3°C/min to 200°C. We compared calculated retention indexes to published values (Jennings and Shibamoto 1980; Davies 1990; Adams 2007).

For diterpene analysis, we weighed ~0.6 g of ground cone powder into a vial and added 4.00 mL of diethyl ether. We capped and mixed vials as above and also allowed them to soak for 7 d. After soaking, we evaporated a 100-μL aliquot of each sample to dryness and then converted diterpenes to their methyl esters with the addition of 100 μL ethereal diazomethane to the residue. We used an Aldrich diazomethane generator (P/N Z411736; Sigma-Aldrich) to make the diazomethane solution using Diazald (N-methyl-N-nitroso-p-toluenesulfonamide; Sigma-Aldrich; Ngan and Toofan 1991). After methylation, we again evaporated each diterpene sample and brought them back up in 100 μL of isopropanol containing carvacrol (0.1 μL/mL; Sigma-Aldrich) as an internal standard.

We also quantitated the diterpene samples on the Hewlett Packard 5890 GC/FID with the same 15-m DB-Wax column. GC conditions remained the same as above, except for the oven profile, which began with an isothermal hold at 170°C for 35 min, followed by a ramp of 3°C/min to a final temperature of 240°C. We calculated diterpene concentrations by comparison with injections of a known concentration of abietic acid (Sigma-Aldrich), methylated as above, also with carvacrol as an internal standard and assuming equal response factors. The USDA Forest Service Forest Products Laboratory in Madison, Wisconsin, supplied small amounts of the other resin acids—as either methyl esters or methylated as above—to determine GC retention times for compound identification. Because of the coelution of palustric and isopimaric acid peaks, these compounds were calculated together. All terpene concentrations were expressed as milligrams of terpene per gram of cone tissue dry weight.

Besides comparing the GC/FID standard and sample diterpene retention times to one another and to the relative retention times of Foster and Zinkel (1982), we performed additional analyses on a Hewlett Packard 1090 high-pressure liquid chromatograph with a diode array detector to confirm the identity of the various resin acids (Kersten et al. 2006). We injected 20 μL of nonmethylated standard solutions (0.6 mg/mL) of levopimaric, palustric, isopimaric, abietic, dehydroxyabietic, and neoabietic acid and selected cone samples (ground tissue in neat ethanol) on a Vydac C_{18} column (7.8 mm × 250 mm) with a mobile phase consisting of MeOH : 1.7% acetic acid (87.5 : 12.5) run isocratically at 1.0 mL/min with the column heater set at 30°C. We monitored UV signals at 240, 268, and 282 nm and used the diode array detector to collect UV data from 190 to 400 nm and compared the resulting UV spectra of each compound to those of other researchers (Zinkel et al. 1971; Kersten et al. 2006; M. Nuopponen, personal communication).

Cone Herbivore Distribution

We collected the cones used for the determination of cone insect distributions haphazardly from many trees throughout the summers of 1988, 1989, and 1998 at five sites in Boulder County, Colorado, at elevations ranging from 1700 to 2950 m: Marshall Mesa (1730 m), Boulder Canyon (1700 m), Bald Mountain (2120 m), Sugarloaf Mountain (2600 m), and Niwot (2950 m). These sites were originally chosen for insect censuses because of both the elevational gradient covered and the apparent range of insect diversity (Y. B. Linhart,

personal observation). We dissected cones and identified insects as larvae or adults or indirectly from frass or exit holes (Hedlin et al. 1981). Because of small sample sizes in 1988, we combined the collections from the summers of 1988 and 1989 (Marshall Mesa, $N = 142$; Boulder Canyon, $N = 675$; Bald Mountain, $N = 158$; Sugarloaf, $N = 179$; Niwot, $N = 115$) and reported them separately from the 1998 data (Marshall Mesa, $N = 41$; Boulder Canyon, $N = 40$; Bald Mountain, $N = 61$; Sugarloaf, $N = 47$; Niwot, $N = 23$).

Chemistry and Herbivory

To correlate herbivore damage with cone chemistry, we counted all of the damaged (closed) and undamaged (open) cones for a total of 25 trees from three sites in the fall of 1999, where terpenoids had been measured earlier that year (Boulder Canyon, 6 trees; Betasso Park, 9 trees; Sugarloaf Mountain, 10 trees). Because of time constraints, we did not count cones on all of the trees at all sites and none at the Marshall Mesa site. Cones infested by any of the three types of insect larvae remained permanently closed, which made them readily distinguishable from unaffected cones, which had opened by this time.

Statistical Analysis

We used SAS (ver. 9.1; SAS Institute 2003) for all statistical analyses and to examine the distributions of all variables to insure that they met assumptions of normality, applying transformations where necessary. A factor analysis on the concentration data of all terpenes using PROC FACTOR with a PROMAX rotation reduced the number of variables. We accepted the first four factors after examining a scree plot of the eigenvalues and used the factor scores of each cone as dependent variables for further analyses. Next, to test for differences in cone chemistry among sites and to determine the chemical variation due to site, tree, and cluster, we performed a nested ANOVA on the factor scores using the PROC NESTED function, with site (four sites) as an outer fixed effect, with tree (10 trees per site) nested within site and clusters nested within tree as random effects (two clusters per tree, with replication provided by two cones per cluster).

To look for differences in monoterpene/longifolene analysis results between samples extracted for 7 or 14 d, we performed separate one-way ANOVAs on each of the 10 compounds using PROC GLM.

To test for temporal and spatial differences in cone insect distributions, we analyzed cone insect count data as contingency tables using the χ^2 statistic (Zar 1999) with the PROC FREQ function. To detect temporal differences, we analyzed each insect species (*C. ponderosae*, moths, and *C. neomexicanus*) separately for year and site. This allowed us to determine whether the distribution of a particular insect at the various sites changed between the two time periods. Differences in insect spatial distributions among the sites were analyzed separately for the collections from 1988/1989 and 1998. This was to see whether the sites differed in their insect profile at any one time.

To determine the relationship between terpenoid chemistry and cone damage, we used the PROC CORR function to test for correlations between the first four chemistry factors versus cone herbivory (proportion of infested cones over the total for each tree) separately at the Boulder Canyon, Betasso, and Sugarloaf sites. Since each of the 25 trees used for this analysis had a single value for herbivory, the factor scores from the four cones per tree were averaged. We analyzed the three sites separately because of the different herbivore assemblages found at each site. Herbivory proportions were arcsine square root transformed to address the issue of interdependence between the variance and mean in a binomial distribution (Sokal and Rohlf 1995).

Results

Terpenoid Analysis

Chemical analyses revealed that most cones contained measurable amounts of all nine monoterpenes (fig. 1*A*). Typically, δ-3-carene represented the most abundant monoterpene, followed by α-pinene, β-pinene, limonene, and myrcene. Many samples did not contain the sesquiterpene, longifolene, or only at low levels when present (fig. 1*A*). Monoterpene/longifolene samples that were soaked for either 7 or 14 d showed no significant differences in their concentrations (all $P > 0.75$).

Abietic acid dominated the composition of the diterpene mixture, which also contained appreciable amounts of neoabietic and levopimaric acids (fig. 1*B*). Samples had relatively high combined amounts of isopimaric and palustric acids, but because of coelution, we could not determine their individual contributions (fig. 1*B*).

We accepted four factors from the factor analysis of cone terpene amounts, which together explained 62% of the total variation in cone chemistry (factor 1 = 22.5%, factor 2 = 18.1%, factor 3 = 13.2%, and factor 4 = 8.2%). We described factors 1 and 2 as diterpene factors because of the heavy loading of several compounds in this class (table A1 in the appendix in the online edition of the *International Journal of Plant Sciences*). In particular, abietic, neoabietic, levopimaric, and isopimaric/palustric acids, the monoterpene limonene, and imbricatocolic acid loaded most heavily on factor 1. The three unknown diterpenes, plus acetylisocupressic and succinylisocupressic acids, loaded the heaviest on factor 2 (table A1). Factors 3 and 4 both represented monoterpene factors as a result of high loadings from δ-3-carene, γ-terpinene, and terpinolene on Factor 3 and α-pinene and camphene on factor 4 (table A1). The lone sesquiterpene, longifolene, showed little variation (low loadings) in any of the four factors.

The nested analysis of the individual cone scores of the four factors showed that only factor 1 varied significantly among the four sites, with positive mean factor scores for both the Sugarloaf and Boulder Canyon sites, which significantly differed from one another and from the negative mean factor scores of both the Marshall Mesa and Betasso sites (fig. 2; table 1). The percent of the total variation explained by trees within sites represented the greatest source of variation for all four factors, accounting for 59.8%–84.7% of the variation, with highly significant results for all of the analyses (table 1). The percent of variation explained by cone cluster was low (1.9%) and nonsignificant for factor 1, but it was significant for the remaining factors and accounted for 6.5%–19.6% of the total variation (table 1).

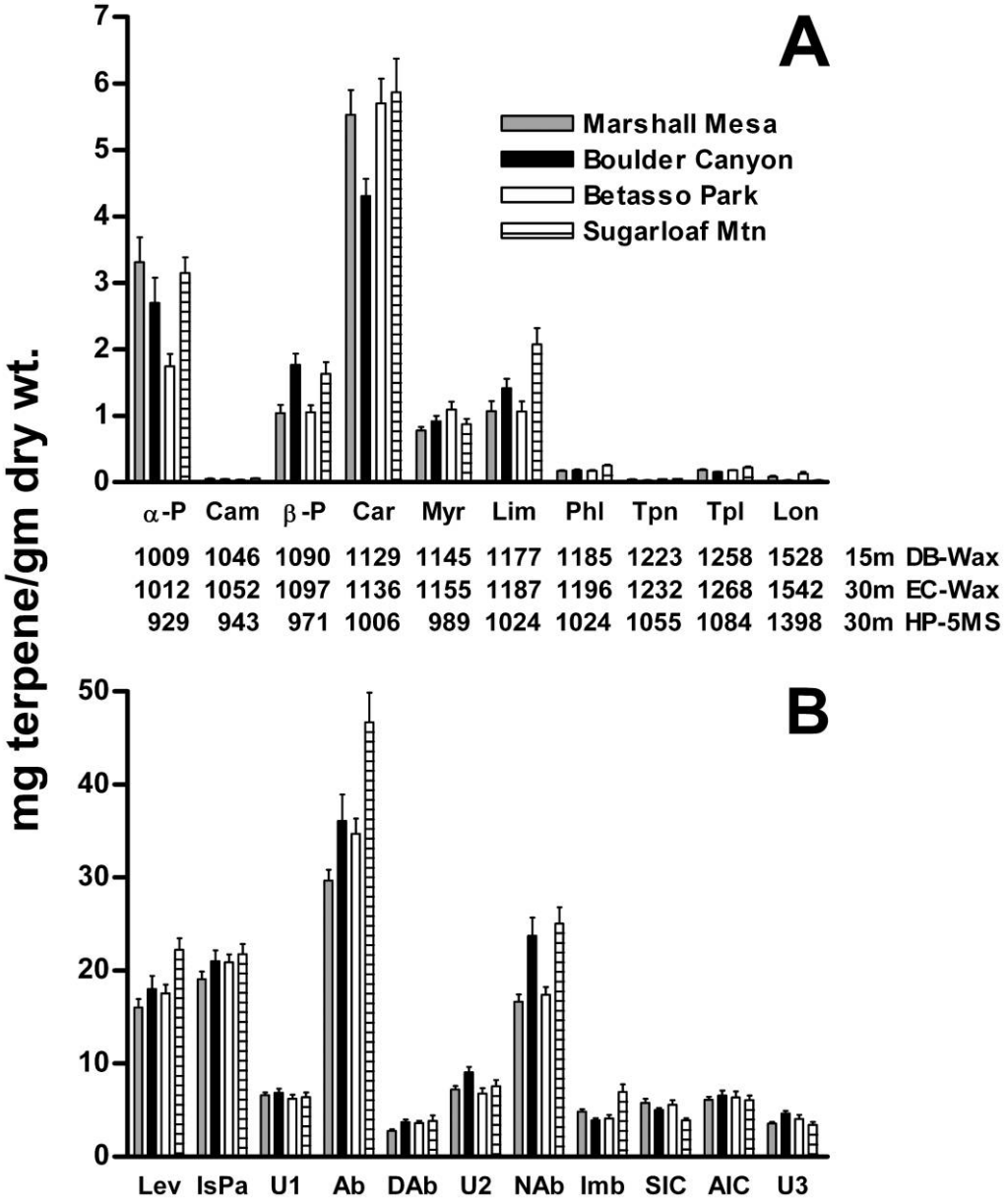

Fig. 1 *A*, Mean (±1 SE) concentrations and linear retention indexes of nine monoterpenes and the single sesquiterpene longifolene in ponderosa pine cones at four different sites. α-P = α-pinene, Cam = camphene, β-P = β-pinene, Car = δ-3-carene, Myr = myrcene, Lim = limonene, Phl = β-phellandrene, Tpn = γ-terpinene, Tpl = terpinolene, and Lon = longifolene. *B*, Mean (±1 SE) concentrations of 12 diterpenes in ponderosa pine cones at four different sites. Lev = levopimaric acid, IsPa = isopimaric/palustric acids, U1 = unknown 1, Ab = abietic acid, DAb = dehydroabietic acid, U2 = unknown 2, NAb = neoabietic acid, Imb = imbricatoloic acid, SIC = succinylisocupressic acid, AIC = acetylisocupressic acid, and U3 = unknown 3.

Cone Herbivore Distribution

The contingency table results indicated that cone insects showed temporal and spatial variation, with differences among years and among sites (fig. 3; table 2). The site by year results revealed highly significant differences for all three groups (*Conophthorus ponderosae*, *Conotrachelus neomexicanus*, and moths) among the two sampling times (table 2). The spatial analyses of insects by site for both the 1988/1989 and 1998 collections also proved highly significant, meaning that there where large differences in the insect profile among sites (table 2).

Chemistry and Herbivory

Correlation results of chemistry factors 1–4 versus percent herbivory yielded two significant results at the Boulder Canyon site. At this site, factors 2 and 4 both showed strong negative correlations with the levels of cone herbivory (table 3).

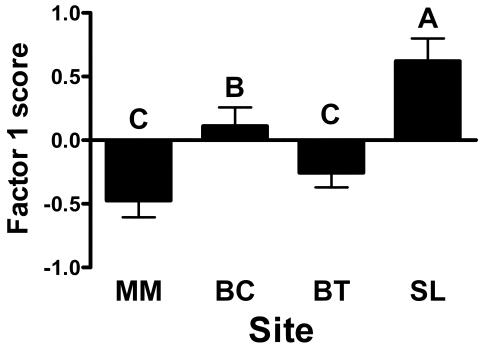

Fig. 2 Mean (±1 SE) factor 1 scores from four different sites. MM = Marshall Mesa, BC = Boulder, BT = Betasso, and SL = Sugarloaf Mountain. Different letters indicate significant differences.

Thus, trees with higher scores for factors 2 and 4 experienced less herbivore damage. We found no other significant correlations at the other sites (table 3).

Discussion

In this study, we demonstrated that ponderosa pine populations exhibit high amounts of variation in their cone secondary chemistry, with most of the variability occurring among individual trees within sites. We also found that cone parasites can vary substantially over time and space, despite the fact that some sites were separated by relatively short distances and that sampling times were 10 yr apart. Finally, we showed that cone chemistry can influence herbivory and that the ratio of mono- to diterpenes may be responsible.

In terms of chemical diversity, most trees contained measurable amounts of 21 of the different terpenoid compounds, including the nine monoterpenes and 12 diterpenes. Smith and coworkers (Smith et al. 1969; Smith 1977, 2000) conducted extensive analyses of xylem oleoresin monoterpenes in populations of ponderosa pine throughout the western United States and delineated five geographically distinct chemical races. The monoterpene patterns in our study corresponded to their region III (Cascade-Northern), an extensive area stretching from the southern Colorado Rockies into Wyoming and Montana and then west to the Cascades in Oregon and Washington and characterized by high levels of δ-3-carene, followed in order by β-pinene, α-pinene, limonene, and myrcene. Our findings matched this general pattern, except for a switch in the amounts of α- and β-pinene (fig. 1*A*). Since Smith assayed tree monoterpenes solely from trunk oleoresin, other work showing that cones contain a higher proportion of α-pinene with less δ-3-carene than trunk resin may explain these differences (Latta et al. 2000).

The profile of diterpenes found in ponderosa cones (fig. 1*B*) matched those of Joye et al. (1969), who also found abietic acid as the dominant diterpene in trunk resin, but differed from those of Fujii and Zinkel (1984), who reported high levels of levopimaric acid (as well as large amounts of abietic and neoabietic acids), and from those of other researchers, who found needle resin consisting of mostly neoabietic and imbricataloic acids (Zinkel and Magee 1991) or neoabietic and isocupressic acids (Wagner et al. 1997). Whether diterpenes in ponderosa also show the same large-scale geographic variation or tissue-specific differences seen in monoterpenes (Latta et al. 2000) needs more systematic study.

We observed significant differences in cone chemistry in factor 1 (a diterpene factor) among the four sites, with Marshall Mesa and Betasso both showing negative mean factor scores, which differed from the Boulder Canyon site's positive mean score, all of which differed from the even higher score of Sugarloaf (fig. 2; table 1). The sites where we tested cone chemistry averaged only ~11 km from one another (range ~4–19 km), distances that are not that far for a wind-pollinated species. Thus, our results show that there can be significant differences in terpene composition over relatively short distances, a pattern also seen for allozyme loci in ponderosa (Linhart et al. 1981).

Besides the several diterpenes that characterized factor 1, the single monoterpene limonene also ranked very high with this factor (table A1). Other workers have identified limonene as an important semiochemical to conifer cone and stem feeding insects, including as a strong oviposition stimu-

Table 1

Nested Analysis Results and Partition of Variance for Factors 1–4 for Terpenes in Ponderosa Pine Cones

	Among sites		Percent variance			
Parameter ($N = 160$ cones)	$F_{3, 36}$	P	Site	Tree	Cluster	Error
Factor 1	2.96	.04	14.44*	69.09***	1.88	14.60
Factor 2	.44	.73	.00	80.69***	6.50**	12.82
Factor 3	1.58	.21	4.29	59.85***	19.63***	16.23
Factor 4	1.39	.26	3.54	84.73***	6.62***	5.20

Note. F and P values are the results of nested ANOVA analyses for among-site differences. Asterisks denote the results of nested ANOVA analyses for among trees within site ($F_{36, 40}$) and among cone clusters within tree ($F_{40, 80}$).

* $0.05 > P > 0.01$.

** $0.01 > P > 0.001$.

*** $P < 0.001$.

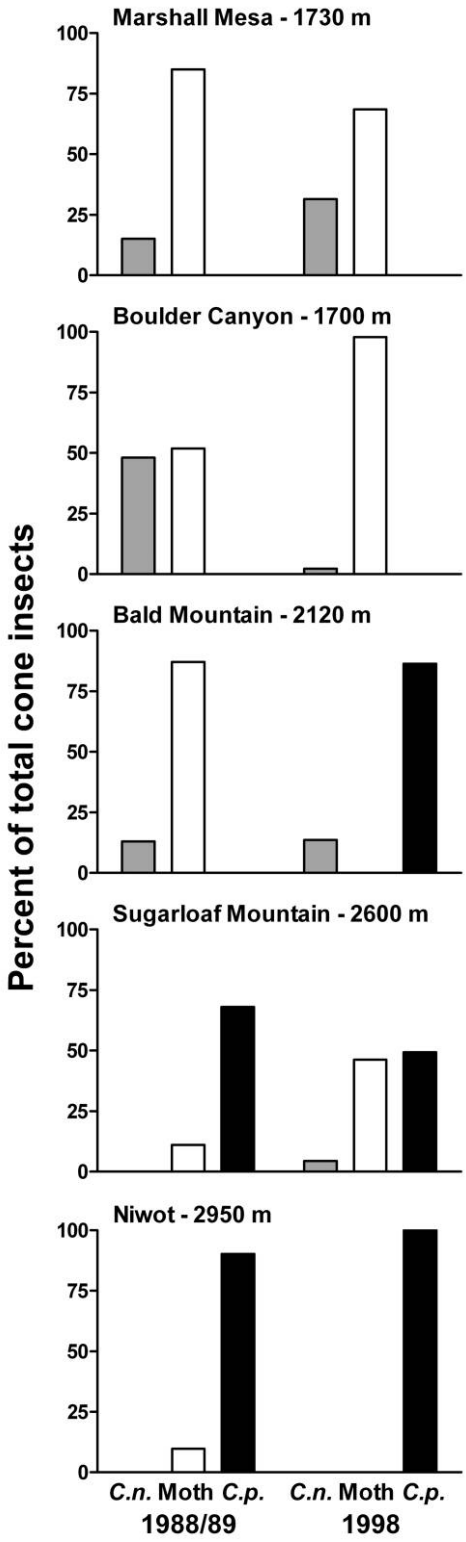

Fig. 3 Percent of total of the different cone insects at five different sites collected in 1988/1989 and 1998. *C.n.* = *Conotrachelus neomexicanus* (gray bars), Moth = *Dioryctria* and *Eucosma* spp. (white bars), and *C.p.* = *Conophthorus ponderosae* (black bars).

lant to the cone moth *Dioryctria abietivorella* (Shu et al. 1997), as a positive indicator for host selection in another stem boring *Dioryctria* (Jactel et al. 1996), and as an attractant for white pine cone beetles *Conophthorus coniperda* (Brauner and de Groot 2006; Miller 2007). The significantly higher factor 1 score we saw at the Sugarloaf Mountain site (fig. 2) seems the most convincing evidence of a relationship between this compound and the cone insect patterns in our study (fig. 3). Part of the high factor 1 score seen at Sugarloaf probably results from the much greater amounts of limonene found in cones at this site (fig. 1*A*). Given its documented attractive effect on a congener (Brauner and de Groot 2006; Miller 2007), the increased levels of limonene at this site may partially explain the much higher prevalence of *Conophthorus ponderosae*. Additional analyses of limonene levels in cones at other sites with high percentages of *C. ponderosae* would help confirm this idea.

In addition to site differences, the majority of chemical variation (60%–85%) occurred among trees within populations (table 1). This means that the cone terpenoid profile of any one tree at a particular site differed from that of its neighbor. Latta et al. (2003) observed a similar pattern of variation in ponderosa resin monoterpenes at the Boulder Canyon site, and similar results have also been reported in analyses of genetic variation measured by allozymes. Hamrick and Murawski (1991) examined plant genetic diversity within and among populations. They found that the within-population component of genetic diversity accounted for an average of 78% of the total polymorphic loci. Consequently, outcrossing forest tree species such as ponderosa pine will typically have the great majority of their genetic variability among individuals within populations and lower variation among different populations for both allozymes and terpenes.

While within-population differences explained the greatest proportion of the total chemical variation, a considerable amount was also seen within trees themselves. Cone clusters within trees accounted for 1.9%–19.6% of the observed variation (table 1), with the highest amount seen in a monoterpene factor (factor 3). The fact that we chose cone clusters regardless of their location on trees might explain these differences. Latta et al. (2000) found significantly more monoterpenes in needles taken from the north side of ponderosas compared with the south. Similarly, Johnson et al. (1997)

Table 2

Contingency Table Test Results for Ponderosa Pine Cone Insect Spatial and Temporal Distributions

Contingency table	df	χ^2	*P*
Conotrachelus neomexicanus (site by year)	4	40.2	<.001
Moths (site by year)	4	12.6	.014
Conophthorus ponderosae (site by year)	4	42.5	<.001
1988/1989 (insects by site)	8	437.6	<.001
1998 (insects by site)	8	100.4	<.001

Note. Moths = *Dioryctria* and *Eucosma* spp.; site = Marshall Mesa, Boulder Canyon, Bald Mountain, Sugarloaf Mountain, and Niwot.

Table 3

Correlation Results for Percent Herbivory versus Factors 1–4 for the Boulder Canyon, Betasso, and Sugarloaf Mountain Sites

	No. trees	Factor 1	Factor 2	Factor 3	Factor 4
Boulder Canyon	6				
R		−.63	−.82	.11	−.86
P		.18	.045	.83	.027
Betasso	9				
R		.13	.20	.28	−.24
P		.73	.60	.46	.54
Sugarloaf	10				
R		−.25	−.00	−.21	.06
P		.49	1.00	.56	.87

found higher levels of monoterpenes in ponderosa needles exposed to sun compared with those that were shaded.

Ponderosa pine experiences considerable temporal fluctuations in its cone herbivores, with all species showing significant differences between years (fig. 3; table 2). For instance, at the Bald Mountain site, the primary cone herbivore switched from moths in the 1988/1989 collection to mostly *C. ponderosae* in 1998. Additionally, moths increased considerably at both the Boulder Canyon and the Sugarloaf Mountain sites, while weevil (*Conotrachelus neomexicanus*) abundance greatly decreased at Boulder Canyon during the 10-yr period (fig. 3). Dormont and Roques (1999) reported similar amounts of temporal variation in the cone insect fauna of the Swiss stone pine *Pinus cembra* during a 5-yr period.

We also found high spatial variation in cone insects at the five study sites (fig. 3; table 2). During both 1988/1989 and 1998, we observed more *C. neomexicanus* and moths at the Marshall Mesa and Boulder Canyon sites and more *C. ponderosae* at Sugarloaf and Niwot. Initially, we thought that elevation may be an important determinant of cone insect distribution (fig. 3), which has been shown in other pine systems (Gworek et al. 2007) and in one case specifically for a *Dioryctria* moth (Dormont and Roques 1999). However, *C. ponderosae* dominated the cones in a ponderosa population at the same elevation as the Marshall Mesa site and only 9 km away, indicating that this species' distribution may be independent of elevation (K. Keefover-Ring, personal observation). Overall, these data support the idea that one factor helping to maintain the chemical diversity in a system may be the population fluctuations of herbivore identity and the associated variation in selection patterns (Shelton 2000, 2004).

The significant negative correlations between herbivory and chemistry factors 2 and 4 at the Boulder Canyon site indicate that cone herbivores at that site, predominately moths and *C. neomexicanus*, may possibly show sensitivity to cone chemistry (table 3). Factor 2 was characterized by heavy loadings from the three unknown diterpenes, plus acetylisocupressic and succinylisocupressic acids, and factor 4 had the highest loadings from the monoterpenes α-pinene and camphene (table A1). In both cases, cone herbivory increased as the scores of the factors decreased (i.e., negative correlations). These results reflect either differences in the deterrence of the terpenes, or the physical properties of cone oleoresin, or a combination of the two. Oleoresin can present a chemical defense through the toxicity of one or more of the terpenes (Elliger et al. 1976; Cates and Alexander 1982 and references therein; Kopper et al. 2005). Thus, the case where herbivory increased with lower values of factor 4 may result from lower amounts of α-pinene and camphene, which made cones more palatable for insect herbivores (table A1). In addition, diterpenes may have a threshold of toxicity, with little effect on herbivores at low doses but a highly toxic effect at high doses (Elliger et al. 1976). The decreasing factor 2 scores leading to increased herbivory are consistent with this explanation (table 3).

Oleoresin physical properties may also affect herbivory. Given that factor 2 is a "diterpene" factor and factor 4 is a "monoterpene" factor, trees with a high score on factor 2 may have resin that is high in diterpenes (a low monoterpene/diterpene ratio) and therefore more viscous resin than a tree with lower scores. Conversely, trees with a high score on factor 4 might have less viscous resin as a result of high amounts of monoterpenes. Other studies have shown the importance of oleoresin physical features, with higher resin flow rates providing more effective protection against bark beetles (Wright et al. 1979; Cates and Alexander 1982) and Abert's squirrels (Snyder 1992) and faster flow to damaged areas (Cates and Alexander 1982; Tomlin et al. 2000). The lack of association between resin composition and intensity of herbivory in other populations shows that the variables we measured may not affect other cone insects—for example, beetles at Sugarloaf—a result not unexpected from a specialist. Also, because of the large fluctuations possible in cone insect populations, effects of plant chemistry on herbivores may not always be detected (Latta and Linhart 1997).

The terpenoid diversity and variation we document within the cones of ponderosa pine are a manifestation of the extensive chemical variability found in this species. The selective forces exerted by cone herbivores may have influenced these chemical patterns. Work with other pine species has shown a tight coupling between tree phenotype and susceptibility to cone insects (Christensen and Whitham 1993). The temporal and spatial variation we found in parasites at the landscape scale, together with their apparent association with host chemistry, illustrates the potential role of cone insects in the evolutionary dynamics of ponderosa pine defensive chemistry.

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