

Linking Deer Browsing and Terpene Production Among Genetic Identities in *Chamaecyparis nootkatensis* and *Thuja plicata* (Cupressaceae)

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To investigate whether differential herbivore browsing reflects genetic variation in plant defense expression, variation in needle terpenes and damage caused by black-tailed deer (*Odocoileus hemionus*) was analyzed on yellow-cedar (*Chamaecyparis nootkatensis*) and western redcedar (*Thuja plicata*). In a 100-genet yellow-cedar population, three genets that were heavily browsed and had extremely low levels of monoterpenes (0–0.36% dry matter), sesquiterpenes, and diterpenes were compared to unbrowsed genets (0.85–3.83% monoterpenes in dry matter). These differences were maintained in individuals protected from browsing, suggesting genetically based variation in constitutive terpene production. In western redcedar, heavily browsed trees had significantly lower total monoterpene concentrations (1.69% dry matter) than lightly browsed trees (3.32% dry matter). One heavily browsed tree expressed no monoterpenes. No differences were found for diterpenes. In both species, the genotypes with extremely low monoterpene concentrations came from the same open-pollinated families.

The survival and competitive capacity of trees can be reduced by herbivores (Barbosa and Schultz 1987; Crawley 1983; Russell et al. 2001). If a trait, such as plant defense, is correlated with herbivore damage and fitness, and is

genetically controlled, then herbivores may act as a selective force on the plant trait (Marquis 1992; Shonle and Bergelson 2000; Simms 1990; Snyder 1993). Secondary metabolites are central to plant defense against herbivores, as herbivores preferentially eat plants with lower levels of such metabolites (Bryant and Kuropat 1980; Cooper and Owen-Smith 1985; Lawler et al. 1998; Suomela et al. 1997). Their production has been well studied, with a special emphasis on the complex chemistry of terpenoids (Bohlmann and Croteau 1999; Langenheim 1994; Seybold et al. 2000; Trapp and Croteau 2001). In conifers, the production of such defenses can actually be induced by herbivory itself or by mechanical wounding, which simulates herbivory (Bohlmann et al. 1997; Lerdaun et al. 1994; Lewinsohn et al. 1991; Litvak and Monson 1998; Marpeau et al. 1989; Steele et al. 1998). But defoliation by herbivores may also reduce the carbon available to produce secondary metabolites (Bryant et al. 1983; Raffa and Smalley 1995; Tuomi et al. 1991). In all cases, if variation in browsing damage is correlated with constitutive or induced variation in chemical defenses, it poses the question of the genetic correlation between damage and defense and of the variation in defense among genotypes. There are few published studies on genetic variation in the browsing resistance of conifers (Dimock et al. 1976; Duncan et al. 2001; Manninen et al. 1998; Mopper et al. 1991; Silen et al. 1986; Snyder 1993). The time span required for most species and experimental constraints such as even herbivore pressure in field tests needed for heritability studies has been a major impediment.

This article investigates the extent to which browsing damage by Sitka and Columbian black-tailed deer (*Odocoileus hemionus sitkensis* and *O. h. columbianus*)

on yellow-cedar (*Chamaecyparis nootkatensis* D. Don Spach) and western redcedar (*Thuja plicata* Donn ex D. Don) is genetically controlled. Monoterpenes are volatile and have been shown to be repellent to deer (Duncan et al. 1994; Elliott and Loudon 1987; Personius et al. 1987; Vourc'h et al. 2002). In contrast, the influence of sesquiterpenes and diterpenes on cervid browsing has been less studied (Danell et al. 1990). In this article, four common garden genetic studies were used to investigate (1) the relationship between browsing intensity and terpene levels, (2) terpene concentrations in protected genets that are normally heavily browsed, and (3) the extent of variation in terpene production among genetic identities.

Materials and Methods

Experiments, Sampling, and Data Analysis

Yellow-cedar. Two yellow-cedar clonal trials were established on southern Vancouver Island, British Columbia, Canada. One hundred genets originating from approximately 30 open-pollinated families from five southern Vancouver Island populations were planted at the Cowichan Lake Research Station (CLRS), Vancouver Island, in 1988. Thirty of these 100 genets were established at Jordan River (JR), Vancouver Island, in 1994. Both trials were randomized complete block designs with six blocks and one rooted cutting (ramet) per genet per block.

Deer browsing was assessed for all individuals at the CLRS and JR sites. In order to compare the terpene content in relation to deer impact, we collected foliage from two ramets at CLRS and one or two ramets at JR from each heavily browsed genet at each site and from five unbrowsed genets at CLRS and

two at JR. Heavily browsed genets were defined as genets with browsing scars on more than 90% of their foliage. Unbrowsed genets were chosen from the same populations and, if possible, from the same open-pollinated families as heavily browsed ones. To control for possible induced effects of deer browsing, ramets from these heavily browsed and unbrowsed genets at CLRS were grown inside a greenhouse inaccessible to deer. After 6 weeks, foliage samples were collected on all ramets to compare terpene concentrations between genets and between growing locations (outdoor and greenhouse).

For the CLRS material, differences in terpene concentrations between growing locations were tested using a nonparametric Wilcoxon signed rank test (Statistix7 2000) and the differences between genets with a Kruskal–Wallis nonparametric one way analysis of variance (ANOVA) (Statistix7 2000). The latter was done for either each growing location separately or for the pooled data, depending on the significance of the Wilcoxon signed rank test used to compare locations. For the JR data, the total concentration of mono-, sesqui-, and diterpenes by genet were graphed, but were not tested because of the small sample size.

Western redcedar. In a western redcedar genecology study, 2500 trees originating from 62 open-pollinated families representing 14 populations from British Columbia were planted in 1996 at Holt Creek (HC), Vancouver Island. Seedlings were protected from deer browsing for the first 4 years using plastic cones. Deer browsing intensity was scored the year after the protection was removed, using a 1–5 scale (1 = no browsing, 5 = completely defoliated).

To investigate the relationship between browsing and chemistry in western redcedar, we sampled foliage from 8 to 10 genets in each of four open-pollinated families (35 genets in total): two families with mean browsing scores greater than 3 and two with mean scores less than 3, representative of high browsing and low browsing, respectively. Using a square root and arcsine transformation to correct for nonnormality (Sokal and Rohlf 1981), transformed terpene concentrations were analyzed using ANOVA (Proc GLM Type III SS; SAS Institute 1999) with family as a fixed source. The terpene concentrations of the two low-browsed and the two high-browsed

families were compared by a linear contrast.

The role of chemistry on western redcedar sensitivity to browsing was further complemented by observation of fifteen 10-year-old western redcedar trees planted as ornamentals at Tlell, Haida Gwaii (HG), British Columbia, where a large deer population imposes a heavy browsing pressure (Martin and Dufresne 1999; Pojar et al. 1980). Of 15 trees, 14 were heavily browsed, while one tree was untouched. Foliage was collected from the unbrowsed tree and from three neighboring browsed trees. The total concentrations of monoterpenes and diterpenes for each tree were determined and results were graphed.

Chemical Analyses

Leaf terpenes were extracted from 1 g of fresh foliage from each plant sample by soaking samples in 10 ml of methanol for 48 h. Leaf material from the same samples were dried at 55°C for 24 h to calculate the dry matter content. After filtration the extracts were stored at –20°C until terpene analyses by gas chromatography (GC). Extracts were analyzed on a Hewlett-Packard 6890 GC equipped with a flame ionization detector. The instrument provides terpene peaks, which were identified using the same method described in Vourc'h et al. (2001). Each peak area was directly proportional to the mass of the corresponding terpene so that the concentration could be calculated.

Correlations between individual terpenes were calculated since profile differences of individual secondary compounds or combinations of compounds of the same group can be important (Connolly et al. 1980; Estell et al. 1998a). All monoterpenes were significantly and positively correlated to one another (Pearson correlation $0.4 < r < 0.9$, $P < .05$), except one minor unknown monoterpene found in some western redcedar at the HC site. Correlations among all sesquiterpenes and among all diterpenes were also significant and positive for yellow-cedars (except for one minor diterpene). This was not the case in western redcedars, where diterpenes and sesquiterpenes were not abundant. Thus all of the related analyses were run using the total concentration of terpene within each chemical group (mono-, sesqui-, and diterpenes for yellow-cedars, and mono- and diterpenes for western redcedars).

Results

Yellow-Cedar

Heavy deer browsing occurred on all ramets from three genets at CLRS (5-8-15; 5 = population number, 8 = open-pollinated family number, 15 = genet number, 5-9-1, and 5-9-2) and two genets at JR (5-8-15 and 5-9-1). Unfortunately genet 5-8-15 was not available for sampling at CLRS because of high mortality due to heavy browsing. Genet 5-9-2 was not planted at JR. None of the other genets showed any sign of extensive browsing (there were no other genets from the 5-9 family). Unbrowsed genets were sampled among the 5-8 and 5-10 families.

Ten major monoterpene, 5 sesquiterpene, and 16 diterpene peaks were found at both the CLRS and JR sites. Three of the monoterpenes were identified (α -pinene, 3-carene, and limonene) and were among those that had previously been identified by von Rudloff (1975). There was no effect of growing location (outdoor or greenhouse) at CLRS for total monoterpene ($P = .675$), sesquiterpene ($P = .281$), and diterpene concentrations ($P = .932$). The genet effect was significant for all terpene groups (monoterpene: Kruskal–Wallis statistic (KW) = 22.22, $P = .001$; sesquiterpene: KW = 21.57, $P = .001$; diterpene: KW = 21.79, $P = .001$) (Figure 1). Genet 5-9-1 produced no monoterpenes or sesquiterpenes and traces of diterpenes at both growing locations. Genet 5-9-2 had less than 0.16% of monoterpenes in dry matter (two trace monoterpenes), no sesquiterpenes, and only traces of diterpenes (Figure 1).

At JR, genet 5-9-1 produced no monoterpenes, but one sesquiterpene and trace amounts of diterpenes (Figure 2). The other heavily browsed genet (5-8-15) had trace amounts of monoterpenes and sesquiterpenes, and considerably less diterpenes than the two other unbrowsed genets (Figure 2).

Western Redcedar

Seven major monoterpene and 10 diterpene peaks were isolated from the trees at the HC and HG sites. Five of the monoterpenes had been previously identified by Vourc'h et al. (2001): α -pinene, sabinene, β -myrcene, limonene, and α,β -thujone. No major sesquiterpenes were found and diterpenes were in smaller quantities than monoterpenes (Figures 3 and 4).

At HC, families 4-2 (4 = population number, 2 = open-pollinated family number) and 22-1, with mean browsing scores of 3.5 and 3.3, respectively, were sampled as representative of heavily browsed families and families 5-6 and 16-3 with mean browsing scores of 2.3 and 2.9, respectively, were sampled as representative of low browsing impact. Analysis of 35 seedlings of these four families showed that the family effect was significant for total monoterpene concentration ($F_{3,31} = 19.3, P < .001$) (Figure 3A). The two heavily browsed families averaged 1.69% (SE = 0.26%) total monoterpenes in dry matter as compared to 3.32% (SE = 0.24%) for the lightly browsed families. The linear contrast for monoterpene concentration between these two family groups was significant ($F_{1,31} = 5.14, P < .001$). One tree from family 22-1 produced no monoterpenes at all, and another from the same family produced only 0.02% in dry matter. There was no significant family effect ($F_{3,31} = 0.56, P = .650$) (Figure 3B) for total diterpenes, nor for linear contrast between the two heavily browsed and lightly browsed families ($F_{3,31} = 0.73, P = .40$).

The unbrowsed western redcedar tree at HG had a much higher monoterpene concentration (7.1%) than the browsed trees (1.5–2.4%) (Figure 4A). This concentration was higher than any of the other western redcedar trees sampled in this study. There was no difference in total diterpene concentrations among all the trees sampled (Figure 4B).

Discussion

Correlation of Browsing Damage with Terpene Concentrations

In all cases studied, unbrowsed or lightly browsed yellow-cedar and western redcedar trees had more monoterpenes than browsed ones. This concurs with previous studies showing that certain monoterpenes deter ungulates (Elliott and Loudon 1987; Estell et al. 1998b; Vourc'h et al. 2002). Sesquiterpenes and diterpenes in yellow-cedars also showed the same correlation with browsing, however, this did not hold true for western redcedars. The relatively higher diterpene concentration in yellow-cedar foliage could enhance antiherbivore defense in this species and explain, in part, why they are less browsed by deer than western redcedar when both species co-

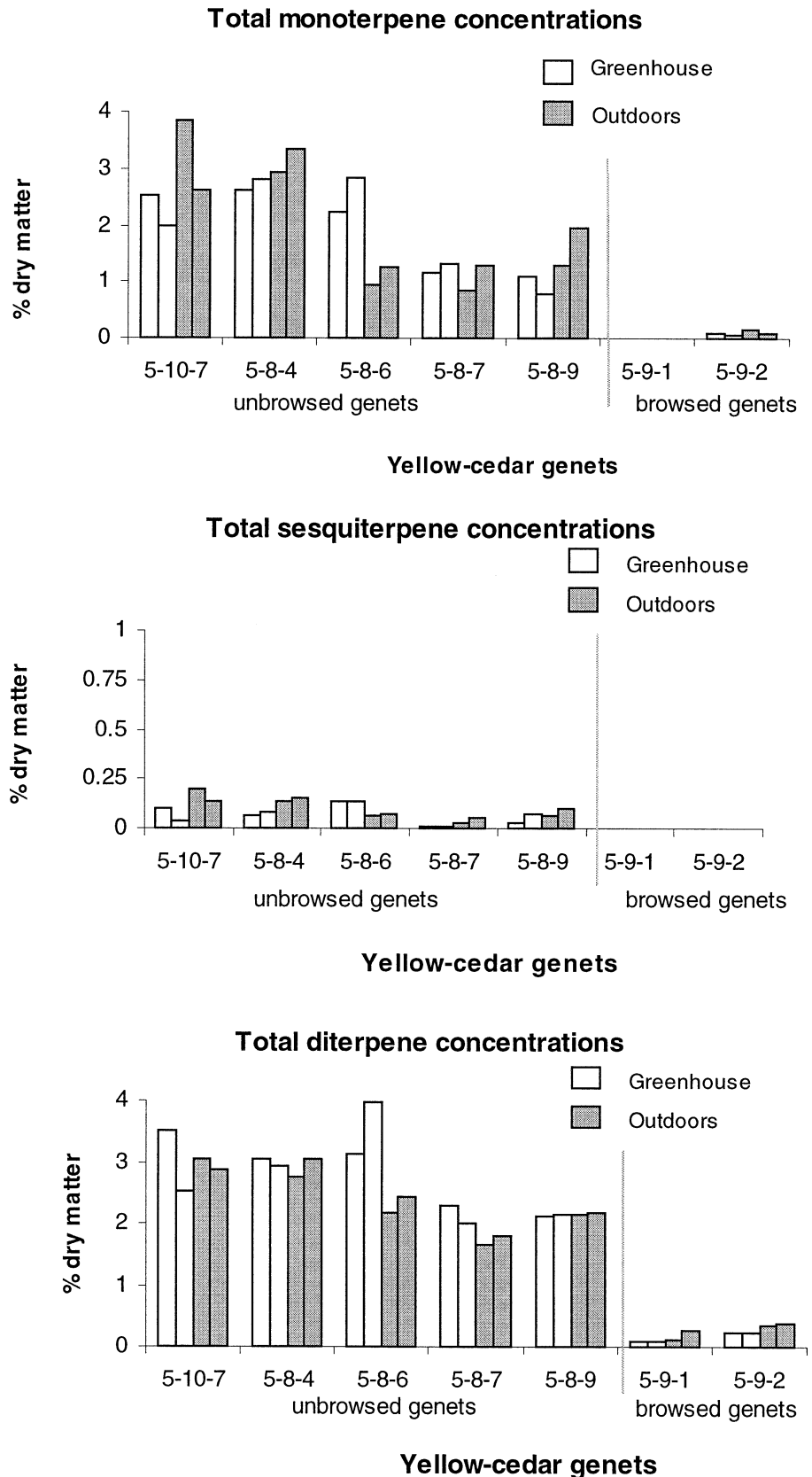


Figure 1. Total monoterpene, sesquiterpene, and diterpene concentrations in browsed and unbrowsed yellow-cedar genets grown at Cowichan Lake Research Station (CLRS), Vancouver Island, Canada. Each column represents one ramet. Genet number reads as follows: 5-10-7; 5 = population number, 10 = open-pollinated family number, 7 = genet number.

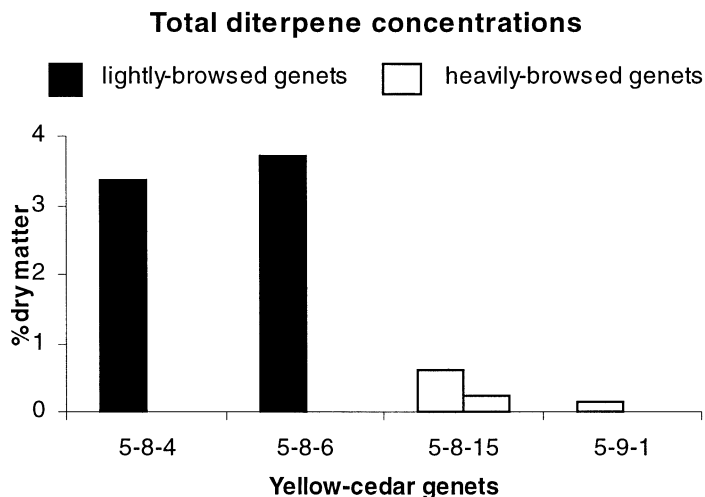
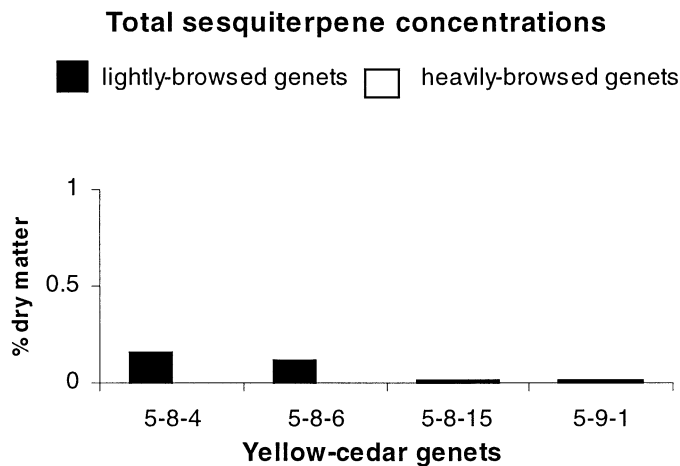
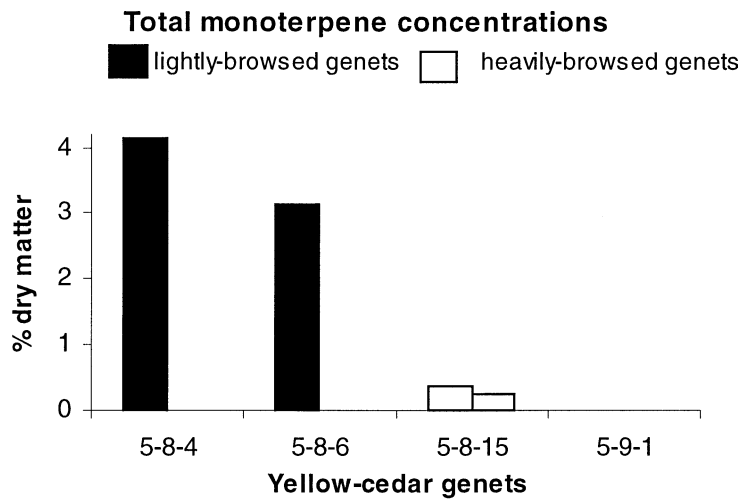


Figure 2. Total monoterpene, sesquiterpene, and diterpene concentrations in browsed and unbrowsed yellow-cedar genets grown at Jordan River (JR), Vancouver Island, Canada. Each column represents one ramet. Genet number reads as follows: 5-8-15; 5 = population number, 8 = open-pollinated family number, 15 = genet number.

occur (Russell J and Martin J-L, personal communication).

Evidence for Genetic Variation in Browse Resistance

Genets suffering extreme browsing had low monoterpene concentrations independent of their growing location and actual exposure to browsing. This suggests genetic differences between plants in their ability to produce terpenes. Previous studies have shown that terpene production can be under the control of a single locus in some species (Marpeau-Bezard et al. 1975; Vernet et al. 1986). The fact that genets most heavily browsed in yellow-cedar and western redcedar stem from a restricted number of families belonging to a single natural population of wind-pollinated trees suggests that very low terpene production is the expression of a rare recessive allele at a gene affecting production directly or affecting a common precursor (Trapp and Croteau 2001). This rare allele may have become established in these populations in the absence of ungulate herbivory pressure. This establishment could be assisted by high population selfing rates, which are common in Cupressaceae (Kuser et al. 1997; Lin et al. 1994; Perry and Knowles 1990; Truesdale and McClenaghan 1998; Xie et al. 1991). Western redcedar has one of the highest selfing rates in conifers, with mean outcrossing rates of 0.35–0.70 (El-Kassaby et al. 1994; O'Connell et al. 2001; Ritland et al. 2001). The western redcedar family with the very low monoterpene genotypes (22-1) originated from a population near the northeastern edge of the species distribution range, and the stand is situated in an isolated bog ecosystem. This could explain both the potential lack of ungulate pressure and the increased selfing rate (Perry and Knowles 1990). Similarly, western redcedars that evolved without any ungulate herbivory pressure on Haida Gwaii were shown to be less defended than mainland ones (Vourc'h et al. 2001).

However, beyond the effect of a major gene, heritability in terpene production has been shown repeatedly (Baradat et al. 1972; Bernard-Dagan et al. 1971; Hanover 1966). Although this study lacks sufficient genetic sampling to properly address this issue, significant variation among the four western redcedar families in monoterpene production, coupled with the correlation of browsing and monoterpene production, is indicative

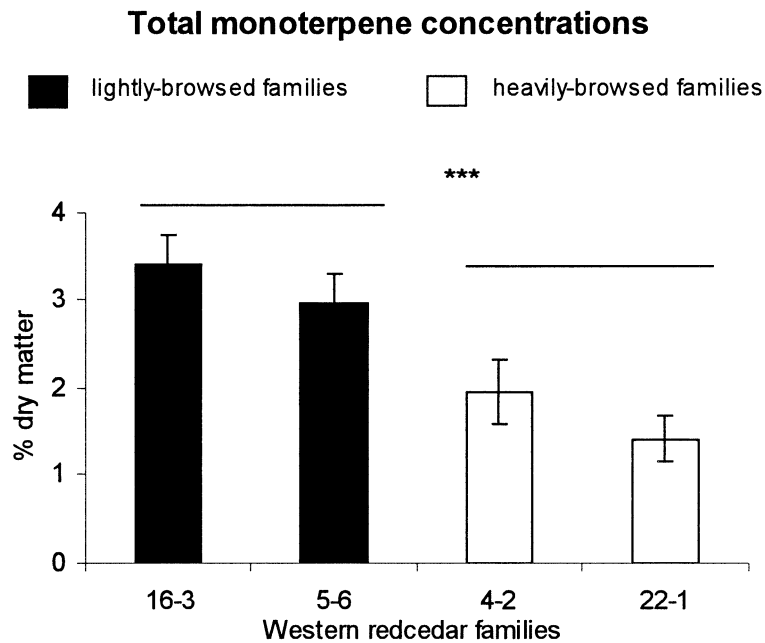
of potential quantitative variation. In a complete survey of deer browsing of all 62 families at HC (data not presented), the percentage of browsed trees per family varied from 6% to 50% (mean 28%). This significant family response to browse resistance has the potential to translate to substantial quantitative variation in monoterpene production if the correlation results from the study reported here can be extrapolated to the entire population.

The HG unbrowsed western redcedar tree had sufficient monoterpene concentration to repel heavy browsing pressure. In fact, the concentration of monoterpenes was 50% greater than the highest concentration in trees sampled at HC.

Results reported in this article on monoterpene variation in western redcedar are in striking contrast to the data presented by von Rudloff and Lapp (1979) and von Rudloff et al. (1988), which show very little variation. However, the authors collected only a few individuals from widely separated western redcedar populations, and might have missed finer scale variability. Evidence of significant genetic variation in browse intensity and monoterpene production shown here, along with results from other studies on restricted fragment length polymorphism (RFLP) (Glaubitz et al. 2000) and isozymes (O'Connell et al. 2001), and on quantitative fitness traits (Cherry 1995; Rehfeldt 1994) shows that western redcedar is less depauperate in genetic variation than was previously thought (Copes 1981; von Rudloff et al. 1988; Yeh 1988).

The remarkable examples in this study show that intraspecific genetic variation in damage by herbivores is significantly correlated with intraspecific variation of mono-, di-, and sesquiterpene content in yellow-cedar and monoterpene in western redcedar. These suggest that significant genetic variation exists on which the evolution of defense mechanisms by natural selection from herbivores can occur. The identification of such strong variation in monoterpene production in cedars opens many opportunities for future research to test fundamental questions in evolutionary theory, including trade-offs between carbon production for energy as opposed to defense mechanisms and the role of mixed mating in population differentiation. Additionally, the material presented in this study is ideal for studying the role of gene

A



B

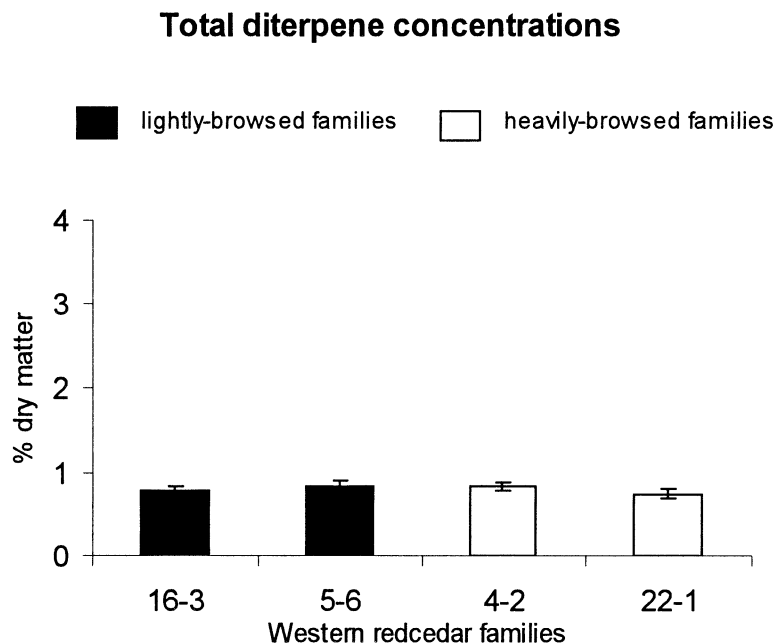
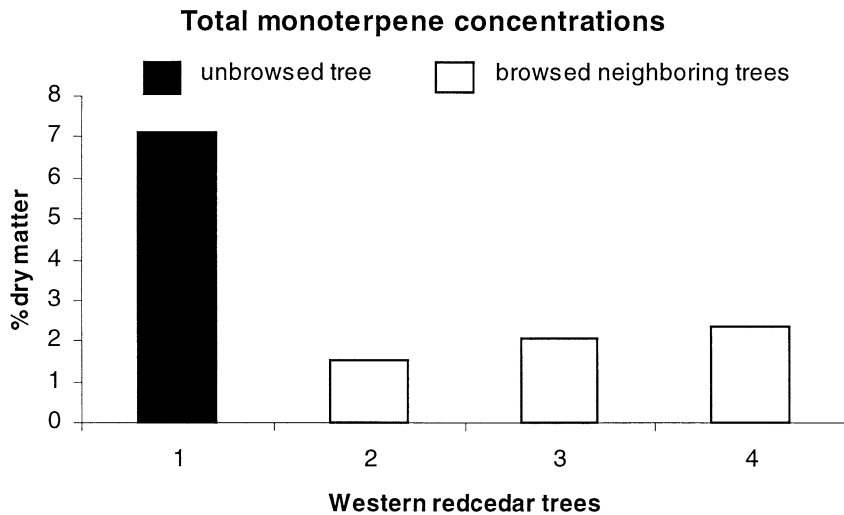


Figure 3. Mean (A) monoterpene and (B) diterpene concentrations in genets from four open-pollinated western redcedar families grown at Holt Creek, Vancouver Island, Canada. Ten, eight, eight, and nine genets were sampled in families 16-3, 5-6, 4-2, and 22-1, respectively. Family number reads as follows: 16-3; 16 = population number, 3 = open-pollinated family number. Vertical bars are standard error. *** $P < .001$.

A



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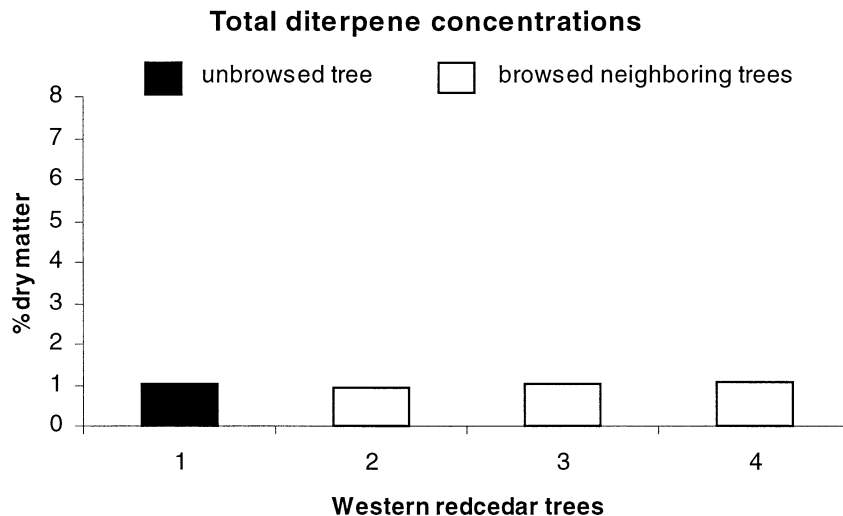


Figure 4. Total (A) monoterpene and (B) diterpene concentrations in browsed and unbrowsed western redcedars grown at Tlell, Haida Gwaii, British Columbia, Canada.

families in terpenoid synthases and how terpenoid synthases and terpenoid formation is regulated.

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