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VARIATION OF PIPERIDINE ALKALOIDS IN PONDEROSA (*Pinus ponderosa*) AND LODGEPOLE PINE (*P. contorta*) FOLIAGE FROM CENTRAL OREGON

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Abstract—We quantified 2,6-disubstituted piperidine alkaloids in *Pinus ponderosa* and *P. contorta* needles from three forest sites in April, June, August, and December. Alkaloids were detected on at least one date in 71% of the ponderosa pine and in 29% of the lodgepole pine trees sampled. Pinidine was the major alkaloid constituent of ponderosa pine, while euphococcinine was the predominant compound in lodgepole pine. For ponderosa pine, total alkaloid concentrations were very low at two sites on all dates. At the third site, concentrations were variable but significantly higher on all dates. Total alkaloid concentrations in previous-year foliage from this site were highest in April, then significantly lower from June through December. Current-year foliage collected in August and December had significantly higher alkaloid concentrations than previous-year foliage on the same dates. Variation in foliar nitrogen concentrations accounted for some of the alkaloid variation in current-year foliage sampled in August.

Key Words—Piperidine alkaloids, pinidine, euphococcinine, foliar chemistry, nitrogen, *Pinus ponderosa*, *Pinus contorta*.

INTRODUCTION

Surveys of piperidine alkaloids in conifers (*Pinus* spp. and *Picea* spp.) have indicated a high degree of qualitative and quantitative variability among species, and among tissues within species (Stermitz et al., 1994; Tallent et al., 1955; Tawara, 1994; Tawara et al., 1993; Todd et al., 1995). Quantitative analyses of young, greenhouse-grown seedlings have described alkaloid variation and

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metamorphosis with age (Tawara et al., 1995; Todd, 1994). For *Pinus ponderosa*, Dougl. ex Laws, pinidine [*cis*-2-methyl-6-(2-propenyl)piperidine] and euphococcinine (1-methyl-9-*nor*-3-granatanone) appear to be final products of biosynthetic pathways involving 1,2-dehydropinidinone, pinidinone, 1,2-dehydropinidinol, and pinidinol as intermediates (Tawara et al., 1993, 1995) (Figure 1).

Our primary objective was to assess the variability of these alkaloids in foliage of trees growing in different forest conditions. We quantified piperidine alkaloids in the two dominant pine species of central Oregon (*Pinus ponderosa* and *P. contorta*, Dougl. ex Loud.) at three sites with differing environmental and silvicultural regimes. Seasonal variation was evaluated by sampling in April, June, August, and December. The August and December collections included current-year needles, in addition to previous-year needles, to assess variation

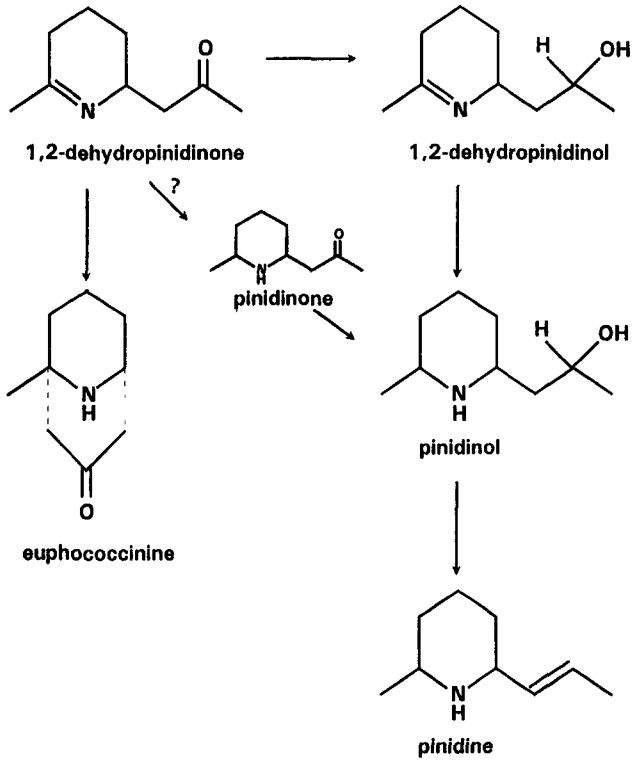

FIG. 1. Hypothetical biosynthetic pathways for piperidine alkaloids found in *Pinus* spp. (Reprinted with permission from *The Journal of Organic Chemistry*, 1993, 58:4816. Copyright 1993 American Chemical Society).

with tissue age. Soil nitrogen availability and total nitrogen concentrations in the foliage collected in August were measured for comparison with alkaloid concentrations. Additional growth-related parameters were measured on the sample trees to characterize differences among the sites and to identify potential correlates to alkaloid production. *Pinus ponderosa* foliage was sampled repeatedly in a 24-hr period at one site to verify that there was no sampling bias due to diurnal variation.

METHODS AND MATERIALS

Site Descriptions. Sites were chosen to represent three distinct plant community types in the pumice region of the Deschutes National Forest, Oregon. Site A is a *P. ponderosa* plantation, approximately 50 years old, unthinned with 28 m^2/ha basal area, located at 4863 km N, 632 km E (UTM zone 10), and 1370 m elevation. *Pinus contorta* is a very minor component of the overstory, and *Purshia tridentata* (Pursh) DC dominates the understory. Site B is a mixed stand of *P. ponderosa* and *P. contorta* at 4856.5 km N, 634 km E, and 1495 m elevation. This site was naturally regenerated after logging 60 years ago, thinned in 1975 and 1992, and burned by wildfire in August 1995, leaving 14.7 m^2/ha basal area and a sparse understory of *P. tridentata* and *Arctostaphylos patula* Greene. Site C is a *P. contorta*-dominated pumice flat with scattered mature *P. ponderosa* at 4844 km N, 610 km E, and 1310 m elevation. It was heavily thinned and pile-burned in 1990 to encourage *P. ponderosa* regeneration. Site C supported 4.8 m^2/ha basal area and a predominantly graminoid understory in the year it was sampled. Annual precipitation (38–43 cm) and site productivity (approx. 3.75 m^3/ha/yr) were equivalent for sites B and C, and slightly lower for site A (38 cm and 3.0 m^3/ha/yr, respectively).

Foliar Sampling. Eight *P. ponderosa* and eight *P. contorta* were randomly selected at each site, usually within a 50-m radius of an arbitrary plot center. Selected trees fit the following criteria: diameter >10 cm; mid-crown within 8.5 m of the ground; and minimal bark damage, beetle attack, dwarf mistletoe, or fire scorch. All three sites were sampled on four dates in 1996: April 25, June 21, August 16, and December 13 (8-, 8- and 16-week intervals), except for site B, which was inaccessible in December. On each date, site A was sampled between 08:00 and 10:30 hr, site B between 10:45 and 13:00 hr, and site C between 13:15 and 15:30 hr. Each tree was sampled within the middle third of the live crown. Two branches from the north aspect and two from the south were clipped with a pole pruner and stored separately (by aspect) in zip-lock bags. The samples were kept on Dry Ice in the field, and at $-36°C$ in the lab until analysis (26–67 days). In April and June, only previous-year needles were sampled; in August and December, both previous-year and current-year needles were sampled.

Diurnal Sampling. Four *P. ponderosa* trees not used for seasonal foliar sampling were selected at site C. All four trees were about 4.5 m tall with nearly full crowns. Four branches in the middle third of the crown on the NNE aspect and four on the SSW aspect were flagged. Two fascicles of needles from each flagged branch were sampled at 20:00 hr on June 27, and at 08:00, 12:00, and 16:00 hr on June 28. For each tree, needles were composited by aspect, and stored in zip-lock bags on Dry Ice in the field and at −36°C for several days in the lab. Skies were clear during the sample period and ambient temperature ranged from 10°C to 20.5°C.

Extraction of Foliage. Our extraction procedure was adapted from Wink et al. (1995), which facilitated large numbers of samples. Preliminary extractions via this procedure and the method of Tawara et al. (1993) provided comparable yields. Samples were processed in randomized sets of eight to minimize systematic error. In a cooler, needles were snipped from the fascicles, diced to <1 cm, and stored in a covered dish less than 30 min before weighing. In the lab, approximately 2 g of needles were accurately weighed into a centrifuge tube, and another 1 g was weighed into a vial for dry weight after 16 hr at 104°C. The 2-g sample was homogenized 30–45 sec in 10 ml of 0.5 M aqueous (aq.), HCl. The homogenizer was rinsed with another 10 ml aq. HCl, and the solutions combined. The tubes were laid on an orbital shaker for 60 min at 100 rpm, then centrifuged for 5 min at 2500 rpm. The supernatant was decanted, and adjusted to pH 11–13 with 6 M NaOH. This solution was loaded on an Extrelut QE prep column (20 ml capacity; EM Separations Technology) and allowed to adsorb for 3–5 min. The column was rinsed twice with 18 ml CH_2Cl_2 and the eluent evaporated at room temperature to <1 ml. The evaporated extract then was transferred to a 1-ml volumetric flask and stored overnight at 3°C. The extract was reduced under a gentle airstream to <0.5 ml before adding 0.5 ml internal standard solution and diluting to volume. The internal standard solution contained 2-ethylpiperidine (98%; ACROS Organics) in CH_2Cl_2.

GC-MS Analysis of Foliage Extracts. An HP5890 gas chromatograph with a J&W Scientific DB-1 capillary column (30 m, × 0.25 mm ID, 25-μm film thickness), an HP5970 mass selective detector, and an HP59970 GC-MS workstation were used for peak identification and quantitation. GC conditions were as follows: injector and transfer line 250°C; initial oven temperature at 85°C programmed at 5°C/min to 135°C; and then 20°C/min to 235°C. A calibration curve for the internal standard versus dihydropinidine was used to calculate response factors as a function of the peak areas of the alkaloids in each sample.

Nitrogen Analyses. The remainder of the diced foliage from the August collection was combined for each *P. ponderosa* tree by equal weights of tissues from N and S aspects, and stored at −36°C. The samples were oven dried at 60°C for 24 hr, ground to pass a 40-mesh screen, and then dried again at 60°C

for 16 hr. Total percentage nitrogen in the foliage was quantified by the Stable Isotope Research Unit, Department of Soil Science, Oregon State University (OSU) in a Dumas combustion apparatus coupled with an isotope ratio GC-MS.

Mineral soil from around each of the 24 *P. ponderosa* trees at sites A–C was sampled to a depth of 15 cm with a sand auger 26 days after the August foliage collection. Two cores from the N aspect and two cores from the S aspect, at 1 m and 2 m from the stem, were combined in ziplock bags, and stored with crushed ice in the field. In the lab, they were stored at 6°C for two days, then homogenized and sieved to <2 mm in size. A seven-day waterlogged incubation of the soil [after Bundy and Meisinger (1994), method 41-2.2.1.2.2] was conducted to provide an index of available N at each site. Soil extracts were analyzed for NH_4 and NO_3 by the Plant & Soil Analytical Lab, Department of Forest Science, OSU, with an Alpchem-RFA 300 rapid flow colorimetric analyzer.

Individual Tree Measurements. Tree diameters were measured at 1.4 m from the ground. The amount of live crown is expressed as a percentage of tree height. The amount of basal area surrounding each sample tree, measured with a 10-factor prism, was taken as an index of overstory competition. Lengths of previous-year branch growth and of previous- and current-year needles were measured on the samples collected in August. Branch lengths are averages of two north and two south aspect branches; needle lengths are averages of four north and four south aspect needles.

Statistical Analyses. No statistical analyses were done for *P. contorta* because of insufficient nonzero data. The following analyses refer to data for *P. ponderosa*. Total alkaloid concentrations are additive quantities of pinidine, euphococcinine, pinidinol, dehydropinidinol, and/or dehydropinidinone expressed as micrograms per gram dry weight. Alkaloid concentrations for N and S aspects were averaged by tree after paired t tests indicated no consistent differences between aspects (Proc MEANS; SAS Institute, Inc., 1996). The effects of site and season on total alkaloid concentrations were tested with a mixed linear model including sampling dates as repeated measures (Proc MIXED; SAS Institute, Inc., 1996). The effect of needle age (current-year vs. previous-year foliage) was tested with August and December data for site C with paired t tests. Foliar N and available N in the soil were compared among sites by analysis of variance, and also tested as covariates for total alkaloid concentration by general linear models that incorporated site effects (Proc GLM; SAS Institute, Inc., 1989). Diurnal effects were tested as repeated measures in SAS Proc MIXED. Responses, i.e., total alkaloid concentrations, were transformed to natural logarithms for homogeneity of variance. Comparisons between means were evaluated with Fisher's protected LSD at $\alpha = 0.05$. The means ($\bar{X}$) reported are back-transformed LS means.

RESULTS

Piperidine alkaloids were detected in 17 of 24 *P. ponderosa* trees (Figure 2), and in 7 of 24 *P. contorta* trees on at least one sampling date. Trees with alkaloids in April usually had alkaloids in June, August, and December. In *P. ponderosa*, pinidine generally constituted 100% of the total alkaloid concentrations, while euphococcinine never exceeded 8%. Intermediate compounds (1,2-dehydropinidinone, 1,2-dehydropinidinol, and/or pinidinol) were detected only at site C in previous-year foliage of one *P. ponderosa* in April, three in June, and two in August; they were detected in current-year foliage in three trees in August. Quantities of intermediates typically were very low, but several trees had 10–35% of total alkaloids as 1,2-dehydropinidinol. Of the *P. contorta*, one tree had distinctly higher alkaloid concentrations, entirely in the form of euphococcinine. Alkaloid profiles for the remaining trees varied considerably, with two trees having small quantities of pinidine. Pinidinone was not detected in either species. None of the intermediate compounds were detected in any trees in December.

Total alkaloid concentrations (dry weight basis) in previous-year foliage of *P. ponderosa* varied significantly, depending on the site and the time of year

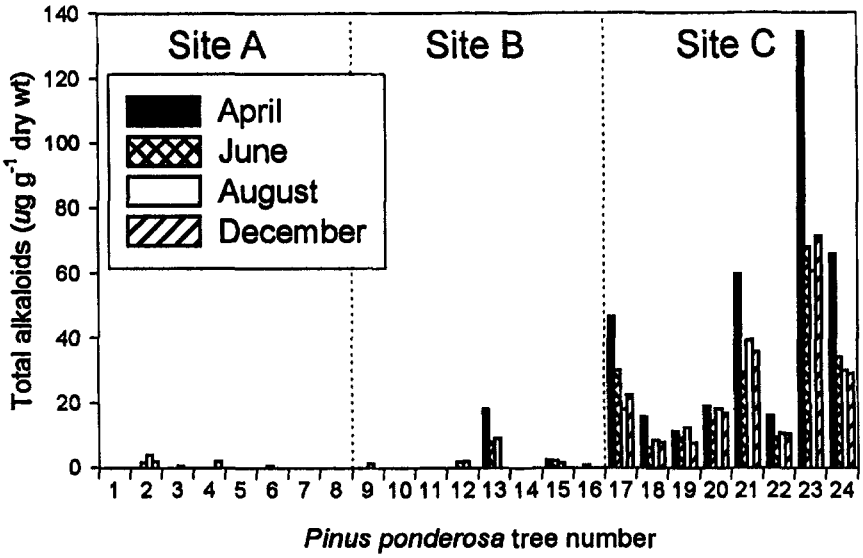

FIG. 2. Total alkaloid concentrations in previous-year foliage of 24 *P. ponderosa* trees from three sites in central Oregon. Mid-crown foliage was collected in April, June, August, and December, excepting site B in December. Bars represent averages for samples from north and south aspects.

($P = 0.010$). At sites A and B, foliar alkaloids were practically nonexistent throughout the year, whereas all trees at site C had piperidine alkaloids in their foliage on all dates (Figure 2). Mean alkaloid concentrations in foliage at site C were higher than at sites A and B on each sampling date (all site A and B $\bar{X} < 1$ μg/g dry wt; all $P < 0.001$). Mean alkaloid concentrations at sites A and B did not differ significantly for any sampling date. Total alkaloids in previous-year foliage at site C decreased from April to June ($P = 0.002$), then remained unchanged from June through August and December (Figure 3). If total alkaloid concentration is expressed on a fresh weight basis, distinctions among sites and dates remain the same. Total alkaloid concentrations of *P. contorta* foliage were <6 μg/g dry wt at all sites and dates, except for one tree at site C with 23–91 μg/g euphococcinine (highest in August).

For logistical reasons, site A was sampled in the morning and site C in the afternoon of each sampling date. To determine whether diurnal variation could explain why trees at site A always had very low alkaloid concentrations (<5 μg/g dry wt) and site C always had much higher alkaloid levels (Figure 2), we sampled four additional *P. ponderosa* trees at site C within a 24-hr period in June. Total alkaloid concentrations in these four trees differed quantitatively, but the variation associated with time of day was insignificant ($P = 0.999$; Figure 4). None of these trees had alkaloid levels at any time of the day that were as low as those observed at site A. Although this analysis does not rule out the possibility that diurnal variation may occur in *Pinus* spp., it does indicate that the large differences in alkaloid concentrations between our study sites were not caused by diurnal variation.

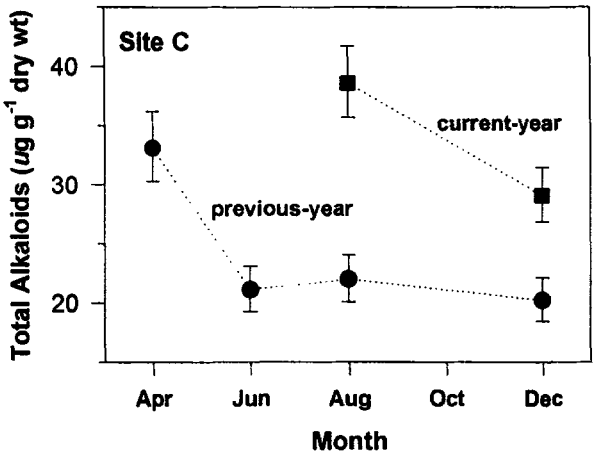

FIG. 3. Total alkaloid concentrations of previous- and current-year foliage from site C. Bars represent standard errors for differences between months, $N = 8$.

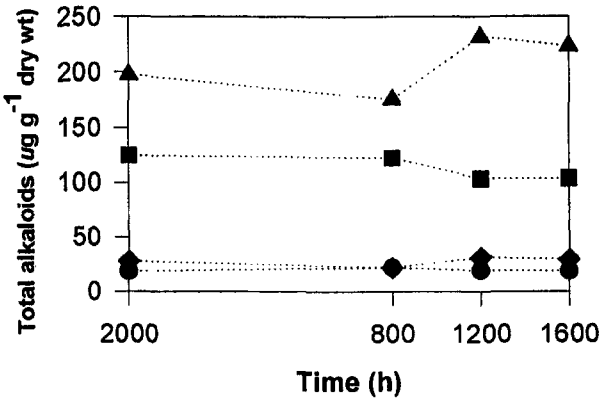

FIG. 4. Diurnal variation in foliar alkaloid concentration of four *P. ponderosa* trees at site C during a 24-hr period in June. Data points are averages for mid-crown samples from north and south aspects.

Total alkaloid concentrations in current-year foliage of *P. ponderosa* at site C decreased from August to December ($P < 0.001$; Figure 3), but alkaloid concentrations in current-year foliage remained higher than previous-year foliage on both dates (August, $P = 0.007$; December, $P = 0.034$). Current-year foliage at sites A and B had alkaloid concentrations of nearly zero in August (and December for site A). *Pinus contorta* was not sampled in December, but, of the five trees with alkaloids in August, all had lower concentrations in current-year foliage than in previous-year foliage.

Total N concentrations in current-year and previous-year *P. ponderosa* foliage collected in August were lowest at site A and highest at site C (Figure 5a). The difference in foliar N between site C and the other sites was stronger

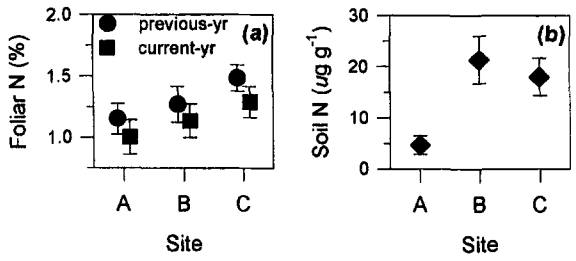

FIG. 5. Means by site for (a) total percent nitrogen in foliage collected in August, and (b) available nitrogen in soil. Bars represent 95% confidence intervals for $N = 8$ sample trees.

for previous-year ($\bar{X}_C:\bar{X}_B$, $P = 0.011$; $\bar{X}_C:\bar{X}_A$, $P < 0.001$; $\bar{X}_B:\bar{X}_A$, $P = 0.144$) than for current-year foliage ($\bar{X}_C:\bar{X}_B$, $P = 0.073$; $\bar{X}_C:\bar{X}_A$, $P = 0.002$; $\bar{X}_B:\bar{X}_A$, $P = 0.107$; Figure 5a). Foliar N was a significant covariate for alkaloid concentrations in current-year foliage across all sites ($P = 0.028$) and in previous-year foliage at site B ($P = 0.007$). After accounting for foliar N effects, alkaloid concentrations in current- and previous-year foliage were still dependent on other factors associated with the different sites ($P < 0.001$ and $P = 0.013$, respectively). Available N in the soil at site A never exceeded 7 μg/g, while sites B and C had higher but comparable levels of available N ($\bar{X}_C:\bar{X}_B$, $P = 0.137$; $\bar{X}_A:\bar{X}_C$, $P < 0.001$; Figure 5b). Available N in the soil was not a significant covariate for total alkaloids in current- or previous-year foliage.

Overall, sample tree sizes were similar across the three sites. Site C had the widest range of tree diameters (Figure 6a) and tree heights (Figure 6b), but

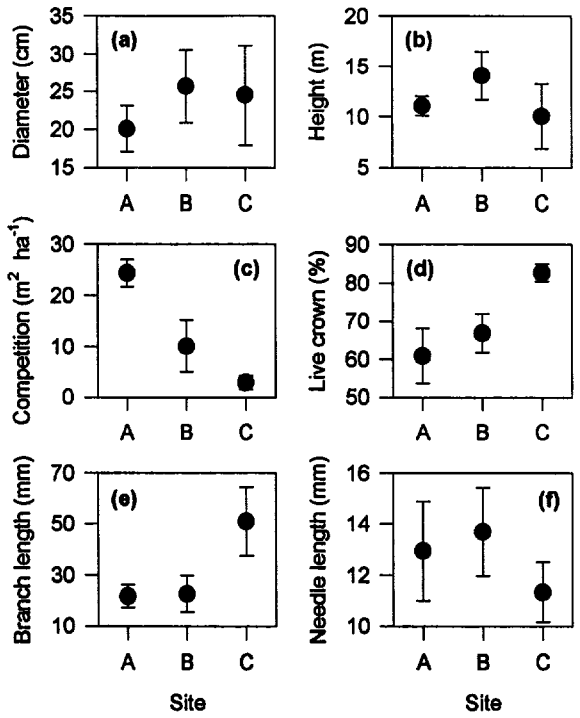

FIG. 6. Means by site for sample tree (a) diameter at 1.4 m, (b) height, (c) competition from neighboring trees, (d) amount of live crown as a percentage of tree height, (e) length of previous-year branch growth, and (f) current-year needle length. Bars represent 95% confidence intervals for $N = 8$ sample trees.

none of the means were distinctly different, so ontogenetic effects were unlikely to confound our comparisons. Overstory competition surrounding the sample trees was significantly lower at site C (Figure 6c), and, correspondingly, live crown percentages were distinctly higher at this site (Figure 6d). Elongation of branches supporting previous-year needles was greater at site C (Figure 6e), but lengths of previous-year (data not shown) and current-year needles (Figure 6f) were somewhat shorter at site C.

DISCUSSION

Species Differences. Piperidine alkaloid concentrations in foliage varied qualitatively and quantitatively between species. Pinidine, the most abundant alkaloid in *P. ponderosa*, was rarely detected in *P. contorta*, and euphococcinine, the most abundant alkaloid in *P. contorta*, was a minor constituent of *P. ponderosa*. These results are consistent with the different biosynthetic pathways proposed for *Pinus* spp. (Stermitz et al., 1994; Tawara et al., 1993, 1995) in which pinidine and euphococcinine are final products. *Pinus ponderosa* at site C apparently utilizes both pathways, but favors the pinidine pathway. Piperidine alkaloids have not been reported previously in *P. contorta*, perhaps because of their sporadic occurrence.

Variation with Season and Age of Needles. The decline in alkaloids in older *P. ponderosa* foliage at site C coincides with new needle elongation in central Oregon, which begins around June and continues through August. This decline could be caused by translocation of alkaloids in previous-year foliage to current-year foliage, or it could reflect breakdown processes or dilution effects associated with carbon deposition in the older foliage. Alkaloid levels tend to be higher in younger leaves (McKey, 1979), but Todd et al. (1995) measured higher concentrations of piperidine alkaloids in older needles of *Picea pungens* Engelm. in March.

Nitrogen Effects. Some of the variation in alkaloid concentrations was explained by differences in foliar N among the study sites. The site with the most alkaloids (site C) also had the highest foliar N levels, and the site with only trace amounts of alkaloids (site A) had substantially lower foliar N levels. Alkaloid and foliar N concentrations at site B were more similar to site A than to site C. Branch growth, percentage live crown, and lack of competition also corresponded closely to alkaloid concentrations, probably reflecting the influence of favorable nutrient status on alkaloid production. The relationship between alkaloid concentrations and foliar N was stronger for current-year foliage, probably because alkaloid concentrations in older foliage had more time to be influenced by translocation, breakdown, or developmental processes. Alkaloid production in lupines also is dependent on plant N content and tissue age (Johnson et al., 1987).

Available N in the soil was not a reliable indicator of foliar alkaloid content. Alkaloids and soil N were low at site A and relatively high at site C, but at site B, alkaloids were low and soil N was high. The recent fire history of site B probably explains the lack of correlation between alkaloids and soil N. Prescribed burning in central Oregon pine stands causes a temporary increase in availability of soil N in the year following a fire (Monleon et al., 1997). Foliar N may not be affected in the growing season following fire, and needle loss may reduce the overall N content of the crown (Landsberg et al., 1984). These factors probably limited alkaloid concentrations in both previous- and current-year needles at site B, even though N availability in the soil was high in September 1996.

Genetic Variation. Genetic differences in *P. ponderosa* may have contributed to the remaining variability in total alkaloids among sites. Trees at site A were planted about 50 years ago, probably from an off-site seed source, while trees at sites B and C were naturally regenerated (Bend-Ft. Rock R.D., Deschutes N.F., records on file). Trees at site A would be genetically most distinct, and this could explain the virtual absence of alkaloids. However, alkaloid concentrations at site B were more similar to site A than to site C, even though trees at sites B and C regenerated from local populations. Yet, site C is 27 km southwest and 185 m lower in elevation than site B, so genetic differences between the sites cannot be discounted. Substantial variability in quinolizidine alkaloids among sites have been attributed to genetic differences within a single lupine species (Wink and Carey, 1994).

Herbivory. Alkaloid concentrations in a variety of plants have been shown to increase or decrease in response to herbivory, depending on conditions such as degree of defoliation, and nutrient or water status of the plant (Brown and Trigo, 1995). Herbivory effects on alkaloids in the Pinaceae are unknown, but defoliation can cause a variety of physiological responses in conifers (Clancy et al., 1995) that could influence piperidine alkaloid production. All three of our study sites were defoliated to some extent in recent years by *Coloradia pandora* larvae, so the alkaloid concentrations we observed may have been influenced by this herbivory. *Coloradia pandora* feed on *P. ponderosa* and *P. lodgepole* until mid-June of alternate years. They finish their larval stage prior to current-year needle elongation in central Oregon, so defoliation directly impacts only older foliage. According to aerial survey classifications, site A was heavily defoliated in 1992 and lightly defoliated in 1994; site B was moderately defoliated in 1990 and 1992; and site C was lightly defoliated in 1994 and 1996 (USDA Forest Service, Forest Insects and Diseases, Region 6, Portland, Oregon). When samples were collected in June 1996, most trees at site C were lightly defoliated (we estimated about 10% of foliar biomass), whereas no defoliation was noted at sites A or B. Thus, any short-term, induced alkaloid response would be expected at site C. This site did have the highest alkaloid concentrations, yet total alkaloid concentrations in previous-year foliage at site

C declined from April to June while the larvae increased in size and defoliation accumulated. Apparently, light herbivory of these trees did not induce piperidine alkaloid production in older foliage. However, the relatively high alkaloid concentrations in current-year foliage at site C could represent translocation or de novo synthesis of alkaloids in younger, more valuable needles, in response to defoliation of the older needles. Pinaceae alkaloids are known to have antifeedant activity against eastern spruce budworm [*Choristoneura fumiferana* (Clem.)] (Schneider et al., 1991) and variegated cutworm [*Peridroma saucia* (Hubner)] (Stermitz et al., 1994).

Summary and Conclusion. This study demonstrated that foliar alkaloid concentrations in *P. ponderosa* can vary greatly from one locality to another, to the extent that piperidine alkaloids may be virtually absent throughout the year from foliage at a particular site. The age of foliage and time of year also affect alkaloids, with younger foliage having higher concentrations. The relative influence of genetics, abiotic environment, and herbivory remains to be determined. Clearly, assessments of piperidine alkaloids in conifers require robust sampling methodologies. Favorable nutrient status measured in terms of foliar nitrogen and growth correlates positively with alkaloid concentrations. Managing *P. ponderosa* at lower stand densities may favor higher alkaloid concentrations by increasing the amount of nutrients available to residual trees. This could be a desirable outcome if these potentially bioactive compounds (Schneider et al., 1991; Stermitz et al., 1994; Tawara et al., 1993) inhibit important herbivores or pathogens.

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