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CAMPHOR FROM JUVENILE WHITE SPRUCE AS AN ANTIFEEDANT FOR SNOWSHOE HARES

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Abstract—One theory in plant antiherbivore defense predicts that slow growing late succession plants like white spruce (*Picea glauca*) make large investments in antiherbivore defenses. Juvenile stages of white spruce in the Yukon, Canada, are rarely browsed by snowshoe hares (*Lepus americanus*), an abundant herbivore, but mature spruce is a highly preferred food. The hexane-soluble fractions of the methanol extracts from juvenile and mature white spruce contain camphor and bornyl acetate. There is four times as much camphor in juvenile spruce as in mature spruce from GC analysis. Plant extracts were added to rabbit chow. Pairs of extracts were offered to hares in choice tests. These tests demonstrated that camphor in the juvenile spruce extracts deterred feeding. Bornyl acetate did not have a clear antifeeding effect.

Key Words—Camphor, bornyl acetate, antifeedant, *Picea glauca*, snowshoe hare, *Lepus americanus*, herbivore, plant defense.

INTRODUCTION

The boreal forest of North America has a generalist mammal herbivore, the snowshoe hare (*Lepus americanus*) which, when in high numbers, imposes a severe browsing impact on trees and shrubs. One theory on plant antiherbivore defense, synthesized by Coley et al. (1985) from earlier statements of Bryant and Kuropat (1980) and Bryant et al. (1983a), suggests that plants which are slow growing make large investments of their resources in antiherbivore de-

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fenses compared to the investments made by fast-growing plants. There has been much work on the antifeeding deterrents to snowshoe hares found in the fast-growing earlier succession shrubs such as birches, willows, and alders (Bryant, 1981; Bryant et al., 1983b, 1985; Reichardt et al., 1984, 1987; Palo, 1984; Palo et al., 1985; Tahvanainen et al., 1985; Clausen et al., 1986). However, there has been little attention paid to the slow-growing later successional plants such as white spruce (*Picea glauca*). In this species, Coley's hypothesis would predict high amounts of antifeedant chemicals because it is slow growing, it retains its needles, and it cannot resprout from the roots if it is ring-barked, as can the fast-growing angiosperms. Another theory (Bryant and Kuropat, 1980; Bryant et al., 1983a) proposes that the juvenile growth phase of boreal trees and shrubs is more heavily defended than the adult phase. This has been demonstrated by these authors for birches, willows, and shrubs but not yet for conifers.

In the Kluane region of southwest Yukon, white spruce is the dominant tree. It is obvious even to the casual observer that juvenile white spruce in that region are hardly touched by snowshoe hares although the trees are easily accessible. (For operational reasons we define "juvenile" here as plants below an arbitrary height of 2 m. Whether size or age are the relevant variables is the subject of other work. Similarly "mature" trees are those over 5 m.) Occasionally one sees a single sapling that has been almost completely browsed, but most juveniles are rarely browsed. On the other hand, mature trees that have fallen during windstorms in winter are clearly favorite food sources, with hares lining up along the fallen tree to feed side by side. Normally these trees, when upright, are out of reach of hares.

Feeding experiments at Kluane in winter (Sinclair and Smith, 1984) showed that mature white spruce was preferred over mature twigs of birches and willows when they were presented together. However, in similar experiments using mature birches and willows but with juvenile spruce, the latter was the least preferred plant type. In experiments where side branches of mature spruce and tops of juvenile spruce were offered together, there was a 10-fold greater amount of mature spruce eaten compared to the juvenile spruce. Despite these clear preference differences shown by hares for the two stages of white spruce, crude chemical analysis showed little difference: crude protein, total phenols, and protein-complexing phenols showed no significant difference, while total resins (ether extractable product) were higher in juvenile spruce but only by a small amount (Sinclair and Smith, 1984). It appeared, therefore, that a specific compound rather than the total resin or phenol content was affecting hare feeding.

This paper describes the analysis of organic extracts from juvenile and mature white spruce. We show that a major constituent of juvenile spruce is camphor, that this is in much lower quantity in mature spruce, and that in feeding trials it is the camphor that deters feeding by snowshoe hares.

METHODS AND MATERIALS

Sample Collection. The distal 30 cm of mature spruce side branches or the tops and side branches of juvenile spruce were clipped while frozen during winter at Kluane. The samples were shipped by air to the University of British Columbia and kept frozen until analyzed.

Extraction and Purification of Secondary Metabolites. The foliage of juvenile white spruce (660 g wet weight) was soaked in methanol (3 liters) at room temperature. After 24 hr, the methanol was decanted and concentrated in vacuo to near dryness. The resulting suspension was partitioned between water (100 ml) and hexane (3 × 300 ml) in a separatory funnel. The hexane layers were combined, dried over magnesium sulfate, and evaporated in vacuo to give a gum (3.0 g) referred to as the "juvenile hexane extract." Foliage of mature white spruce (660 g wet weight) was treated in an identical manner to generate the "mature hexane extract" (3.2 g).

[^{1}H]NMR spectra of the juvenile hexane extract (Figure 1) and the mature hexane extract (Figure 2) were run in $CDCl_3$, with TMS as an internal standard, on a Varian XL300 spectrometer.

Fractionation of the juvenile hexane extract was achieved by flash chromatography (Still et al., 1978) on silica gel 60 (230–400 mesh) using a step-gradient elution scheme involving mixtures of petroleum ether (40–60°C) and diethyl ether (200 ml each of 0, 10, 20, 50, 70 and 100% of petroleum ether in diethyl ether; 50-ml fractions were collected). The separation was monitored via silica gel thin-layer chromatography (TLC) of the collected fractions.

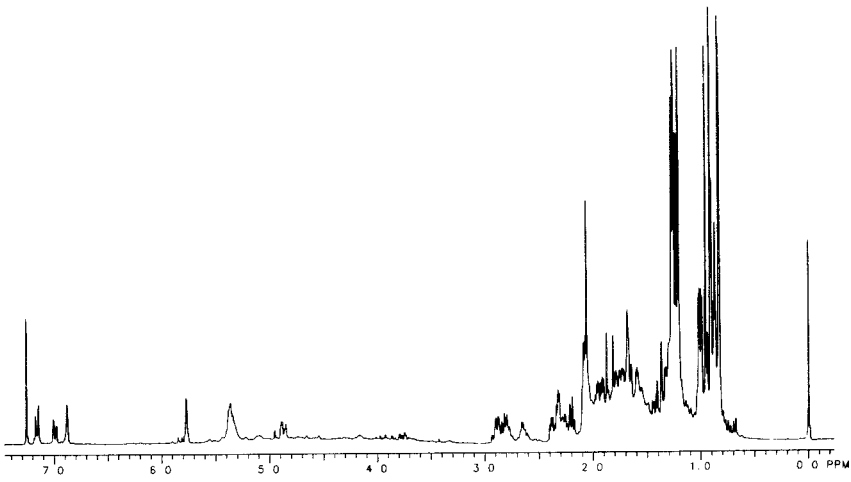

FIG. 1. [^{1}H]NMR (300 MHz) of juvenile hexane extract.

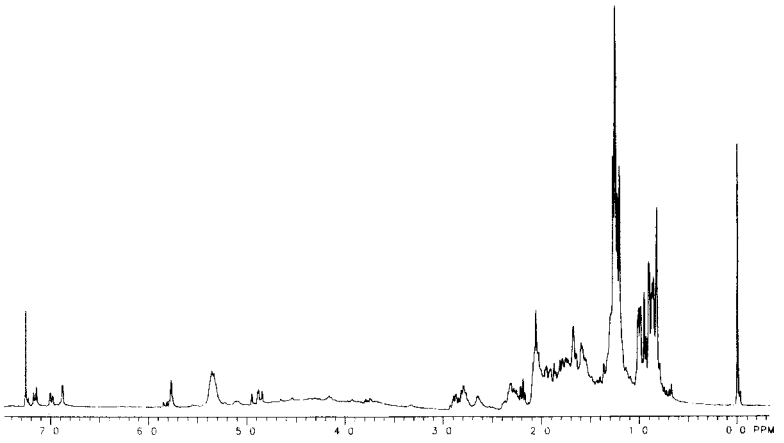

FIG. 2. [^{1}H]NMR (300 MHz) of mature hexane extract.

Preparation of Bioassay Samples. A "juvenile hexane extract with camphor" sample was prepared first by extracting the foliage of juvenile white spruce (660 g wet weight) as described above to get a juvenile hexane extract. This extract was then fractionated via flash chromatography as described above. All the fractions devoid of camphor by TLC analysis were recombined and concentrated in vacuo to give the "juvenile hexane extract without camphor" sample. A second batch of juvenile white spruce (660 g wet weight) was extracted and chromatographed in an identical fashion. All of the fractions from the second batch, including the ones containing camphor, were combined and concentrated in vacuo to give a "juvenile hexane extract with camphor control" sample. Similar procedures were used to prepare "juvenile hexane extract without bornyl acetate" and "juvenile hexane extract with bornyl acetate control" samples.

Samples for bioassay were dissolved in diethyl ether (300 ml) in a 1-liter round bottom flask. Rabbit chow (330 g dry weight) was added to the ether solution, and the solvent was removed in vacuo on a Buchi rotary evaporator to give "treated" chow. White spruce is 50% water, so the amount of rabbit chow used was equivalent to the dry weight of spruce from which the extracts were obtained.

Quantitative Analysis of Camphor and Bornyl Acetate. Quantitative analysis of camphor and bornyl acetate was carried out via analytical gas–liquid chromatography on a Hewlett Packard 5830A gas chromatograph using a 2 m $\times$ 2 mm (ID) stainless-steel column packed with 3% Carbowax 20 M on Chromosorb W. Helium, delivered at a flow rate of 10 ml/min, was used as a carrier

gas. Individual runs were temperature programmed from 100–180°C at a rate of 5°C/min. A known quantity of borneol was added to the methanol extracts of white spruce as an internal standard. The methanol extracts were then treated as described above to get juvenile and mature hexane extracts for GC analysis. Standard calibration curves were prepared for camphor, bornyl acetate, and borneol using weighed samples of authentic material. A Hewlett Packard 18850a computing integrator was used to quantify peak areas. The identity of peaks in the juvenile hexane extract and in the mature hexane extract was confirmed by GC-MS on a Kratos MS80 instrument.

Bioassay of White Spruce Extracts. The general procedure used approximately 80-g amounts of rabbit chow treated with extract. These were offered in bowls to the hares in separate cages overnight. The extracts were offered in pairs so that the animal could make a choice in its feeding. Weights of each chow type were recorded before and after feeding. This procedure was repeated for a minimum of four nights with the location of the two bowls in each cage switched each night to avoid location biases by the hares.

All statistical tests on the amount eaten used the nonparametric Wilcoxon matched-pairs signed ranks test (Siegel, 1956).

RESULTS

Isolation of Secondary Metabolites. The [^{1}H]NMR spectra of juvenile hexane extract (Figure 1) and mature hexane extract (Figure 2) showed significant differences. In particular, there were a series of intense high field methyl resonances present in the juvenile extract spectrum that were not present in the mature extract spectrum. We suspected that the secondary metabolite(s) responsible for the antifeedant activity of the juvenile hexane extract were also responsible for the additional high-field methyl resonances in the [^{1}H]NMR spectrum of the juvenile hexane extract.

The flash chromatography carried out on the juvenile hexane extract gave, in the order of elution, pure bornyl acetate, pure camphor, and a complex mixture of diterpenoic acids, that were only partially characterized. Camphor and bornyl acetate were shown to be identical to authentic samples by [^{1}H]NMR, [^{13}C]NMR, MS, TLC, and GC comparisons. It was possible to attribute all the additional high-field methyl resonances in the [^{1}H]NMR spectrum of juvenile hexane extract (Figure 1) to protons in either camphor or bornyl acetate.

Quantitative Determinations of Camphor and Bornyl Acetate. GC analysis showed that in juvenile white spruce the concentrations of camphor and bornyl acetate were 2.52 and 1.26 g/kg wet weight of foliage, respectively. In mature white spruce, the concentrations of camphor and bornyl acetate were 0.65 and

0.42 g/kg wet weight of foliage, respectively. Thus, we found that there was roughly four times as much camphor in juvenile white spruce foliage as there was in mature foliage.

Bioassay of Secondary Metabolites: Experiment 1. Camphor was added to chow at a concentration of 0.5 g/330 g of chow. This was approximately one fifth the concentration in an equivalent dry weight of juvenile white spruce. The control was ether-treated chow. The results for the amounts eaten are given in Table 1. The animals significantly avoided the camphor-treated chow ($P < 0.001$), and they ate approximately five times as much of the control chow compared with the camphor chow.

Experiment 2. The mature hexane extract was added to chow at the same concentration as that of the camphor chow (0.5 g extract/330 g chow). This concentration of mature hexane extract represented a sixth of that found in the plant. The dilute mature hexane extract was offered to hares with the camphor chow (Table 1). Figure 3A shows that the hares preferred the mature hexane

TABLE 1. AMOUNTS OF RABBIT CHOW TREATED WITH WHITE SPRUCE EXTRACTS EATEN BY SNOWSHOE HARES IN CHOICE TESTS[a]

Extract in chow	Amount eaten	
	Mean weight ± SE/day (g)	% of total
Experiment 1		
Ether solvent	51.5 ± 5.7	84.3
Camphor	9.6 ± 3.5	15.7
Experiment 2		
Dilute mature hexane extract	64.1 ± 4.3	86.3
Camphor	10.2 ± 3.2	13.7
Experiment 3		
Normal mature hexane extract	33.4 ± 5.6	46.2
Camphor	39.0 ± 5.7	53.8
Experiment 4		
Normal mature hexane extract	44.2 ± 5.4	82.8
Normal juvenile hexane extract	9.2 ± 3.8	17.2
Experiment 5		
Juvenile hexane extract without camphor	43.8 ± 4.9	75.1
Juvenile hexane extract with camphor control	14.5 ± 3.5	24.9
Experiment 6		
Juvenile hexane extract without bornyl acetate	35.1 ± 4.7	59.8
Juvenile hexane extract with bornyl acetate control	23.6 ± 3.1	40.2

[a] Four replicate runs were made for each experiment.

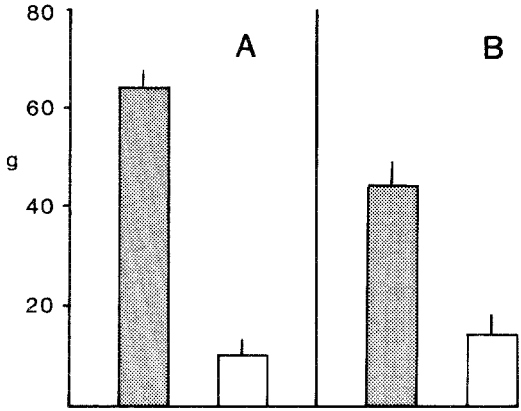

FIG. 3. The mean amounts of rabbit chow eaten per day by snowshoe hares. (A) Chow treated with mature hexane extract (shaded) and camphor (open) both at 0.5 extract/330 g chow. (B) Chow treated with juvenile hexane extract without camphor (shaded) and with camphor (open). Verticle lines are one standard error.

extract over the camphor chow ($P < 0.001$), eating approximately six times as much of the former.

Experiment 3. The mature hexane extract was added to chow at the concentrations found in the natural plant (3.2 g extract/330 g chow). This was offered to hares with the camphor chow. Table 1 shows that the hares did not show a preference between the two, eating similar amounts of both. The mature spruce extract contained 0.43 g camphor in 330 g of chow (from the quantitative analysis above), slightly less than that in the camphor-treated chow. The result of no significant choice is, therefore, to be expected since the camphor concentrations in the two choices were nearly the same.

Experiment 4. In experiment 3 the concentrations of the mature hexane extract and the camphor were markedly different, and the former was noticeably stronger smelling. To balance the secondary metabolites on offer, juvenile hexane extract was offered to hares at the same concentration as that of the mature hexane extract (3.2 g extract/330 g chow). The two hexane extracts were offered together. Table 1 shows that in this situation preference of the mature hexane extract was reestablished ($P < 0.005$).

Experiment 5. To test whether camphor was the major antifeedant in juvenile spruce, camphor was separated from the remainder of the juvenile hexane extract by flash chromatography as described above. In a portion of the remainder, camphor was replaced; in the other portion, camphor was excluded. This procedure controlled for all extraction processes. Juvenile hexane extract with camphor was tested against the same extract without camphor (Table 1). Figure

3B shows that hares preferred the juvenile hexane extract that did not contain the camphor ($P < 0.005$), eating three times as much of this than the extract with camphor.

Experiment 6. The other substance found largely in juvenile spruce was bornyl acetate. The procedure for treating chow with this compound was the same as that for camphor in experiment 5. Thus juvenile hexane extract with bornyl acetate in chow was offered to hares together with chow containing similar extract without bornyl acetate. Although the hares ate more of the extract without bornyl acetate, the result was not significant ($P > 0.05$).

DISCUSSION

The first feeding experiment showed that hares avoided camphor when it was added to chow. But it is possible that hares were simply avoiding any odoriferous substance, so that ether chow was not a proper control. Experiment 2 addresses this aspect by using a concentration of mature spruce extract similar to that of camphor. The avoidance of camphor in this case is more convincing; it appears to be the camphor itself that is being avoided.

The lack of preference shown by hares in experiment 3 could be due to either the similar quantities of camphor present in the two choices or to the higher concentration of other secondary metabolites in mature spruce (2.77 g extract/330 g chow). These other secondary metabolites could counteract the avoidance of camphor (0.5 g camphor/330 g chow). Experiment 4, therefore, was designed to balance the other secondary metabolites in mature and juvenile spruce, leaving only the camphor as the difference between them. Again in this case the animals avoided the juvenile hexane extract containing the camphor. Finally, experiment 5 was designed to show that camphor was the active antifeedant compound by comparing juvenile hexane extract with and without camphor. Extraction procedures were kept identical to avoid hidden biases. The hare preferences confirmed that it was the presence of camphor in the juvenile hexane extract that caused them to avoid it.

Bornyl acetate did not produce a significant antifeeding response in the hares (experiment 6). However, the results suggested there might be some avoidance and further testing is needed. In any case, avoidance of bornyl acetate was much less than avoidance of camphor.

In conclusion, we show that camphor, a specific compound in white spruce, acts as a major antifeedant for snowshoe hares. Camphor occurs at four times the concentration in juvenile spruce (<2 m high) compared to that in mature spruce (>5m high). Another compound, bornyl acetate, did not show significant antifeedant properties. Our results support the hypothesis of Coley et al. (1985) that late-succession slow-growing plants should be strongly defended

against herbivores. The results also support the growth-stage hypothesis of Bryant and Kuropat (1980) that early growth stages are more heavily defended than mature stages.

For these conifers, one evolutionary explanation could be that defense is against smaller mammals such as hares because the presence of camphor is associated with plant height. In addition, Sinclair and Smith (1984) found that preference for spruce tops at 3 m height was intermediate between preferences for spruce at <2 m and >5 m high. Defense against either insects or large mammals (browsing mastodons and giant ground sloths were present in these ecosystems less than 12,000 years ago; Dreimanis, 1968) would not be tied to height.

However, we note that the angiosperms, such as alder, also show a diminution in chemical defense in mature stages, but these stages are usually available to browsing hares. This suggests an alternative explanation that the lower defense of mature twigs is due to fundamental physiological changes in the plant rather than a relaxation of predation. What now remains is to see how camphor content is related to size and age of the spruce plant.

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