ABSTRACT

CHEMICAL ANALYSIS OF SPECIES RELATIONSHIPS
IN NORTHEAST AMERICAN SPRUCES

by

Ronald C. Wilkinson

Analyses of phenolic compounds in the foliage and of monoterpenes in cortex oleoresin were done to study the chemical relationships among white spruce (Picea glauca), black spruce (Picea mariana), and red spruce (Picea rubens).

Foliage samples used for the analysis of phenolic compounds were obtained from 1-year-old internodes of lateral branches of 10- to 20-year-old trees growing in test plantations in Canada and Southern Michigan. Both intraspecific and interspecific variation were examined. The occurrence of hybridization in many sources of red spruce and black spruce provided an opportunity to evaluate the use of phenolic compounds for the study of natural hybridization and introgression.

Two groups of phenolic compounds, simple phenols and polyphenols, were used for making species comparisons. Most of the 162 compounds detected by paper chromatography were common to all three species. Twenty-five compounds

showed patterns of variation associated with species and served to distinguish white, red and black spruce. White and black spruce were the most similar in their phenolic composition. Red spruce was more similar to black spruce than to white spruce. Geographic variation in the phenolic composition of red and black spruce was associated with hybridization. Geographic variation in the phenolic compounds of white spruce was less than in the other two species and no distinct patterns were apparent.

Pure red spruce from North Carolina was less similar to black spruce than sources which overlap the range of black spruce. Known F_1 hybrids were equally similar to both species and contained compounds specific to both.

On the basis of the occurrence of two compounds normally found only in black spruce and on total chemical similarity in both simple and polyphenols, hybridization and the degree of introgression could be determined in sources of red spruce. Hybridization in black spruce could be determined only on the basis of total polyphenols.

Chemical comparisons between samples of the same source revealed much tree-to-tree variation in phenolic compounds. Both quantitative and qualitative variation in phenolic compounds was found between age of foliage, fresh and dry foliage, and season of collection. These facts must be considered in genetic studies using these compounds.

The cortical oleoresin of white spruce contained nine monoterpenes as detected by gas-liquid chromatography. The concentrations of β-pinene, limonene, β-phellandrene, and 3-carene differed between geographic sources and exhibited a distinct east-west variation pattern. A cluster analysis utilizing the major monoterpenes also indicated that white spruce from the east differs in terpene composition from western sources.

On the basis of frequency distributions, α- and β-pinene appear to be under multiple gene control. The distributions of 3-carene, myrcene, limonene, and β-phellandrene were abnormal which may indicate control by few genes. Simple correlations between individual monoterpenes were inconsistent with those of other species except for a high positive correlation between 3-carene and terpinolene.

The monoterpenes of red spruce did not vary significantly between geographic sources despite the hybridization with black spruce found in many of the sources examined. The concentrations of six monoterpenes differed between red spruce and black spruce from Michigan. Two sources of red spruce which resembled black spruce the most in phenolic content and growth characters showed no greater resemblance to Michigan black spruce in terpene content than other sources of red spruce. Red spruce was more similar to black spruce in quantitative terpene

content than to white spruce. The close phylogenetic relationship between black spruce and red spruce was confirmed.

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INTRODUCTION

The application of chemistry to systematic problems in plants is becoming increasingly popular. Alston and Turner (1963a) point out that since 1950 plant taxonomy has entered a phase of biochemical investigation which was made possible by the development of rapid and simple analytical techniques such as chromatography. The choice of chemicals as taxonomic characters is prompted by the desire to observe characteristics close to primary gene action. Chemicals also often appear to exhibit a greater stability than many other characteristics. Furthermore, chemical data are specially beneficial when morphological differences are subtle.

Biochemical approaches to systematics are capable of sufficient refinement to detect not only species but also the degree of hybridity occurring in hybridized populations (Alston and Turner, 1962 and 1963a; and Alston and Hempel, 1964). A biochemical approach therefore seemed desirable in the genus Picea, especially in those species occurring in northeast America. The genus Picea is represented by three species of spruce in the northeastern United States and Canada. White spruce (Picea glauca) and black spruce (Picea mariana) are far ranging

species and occur transcontinentally from New England across boreal Canada into the interior regions of Alaska (Figures 1 and 2). Red spruce (Picea rubens) occupies a more restricted range and is confined to cool, moist habitats from the southern Appalachian Mountains to the Maritime provinces of eastern Canada. An outlying population of red spruce also occurs in southern Ontario (Figure 3).

All three species are economically important, but their genetic improvement and silvicultural potential has not been fully evaluated due to a lack of knowledge concerning their taxonomic relationships. Although 35 to 40 distinct species of spruce are recognized, Wright (1955) in analyzing the geographic distribution, morphological characteristics, and species crossability in the spruces concluded that morphological differentiation has been slight and the range of variation in any one character is small. He also concluded that isolating mechanisms in the genus have largely been geographic and that the removal of geographic barriers has often resulted in hybrid formation and introgression.

The processes of hybridization and introgression appear to have played an important role in the evolution of red and black spruce. Red spruce on the upper slopes of the Appalachian Mountains are distinct from black spruce growing in boreal regions of Canada and bogs in

the Lake States. However, variable types are found in the overlapping regions of the ranges of these two species. Character association analyses indicate that black and red spruce are two species which were isolated in the past but have come together since the Pleistocene and are hybridizing (Morgenstern and Farrar, 1964). This conclusion is supported by reports of hybrids obtained by artificial crosses (Wright, 1955).

White spruce more closely resembles spruce species of northwest America than it does red or black spruce which are considered to be closely related remnants of a more ancient migration than that giving rise to western species of spruce (Wright, 1955). However, white spruce is sympatric with both black and red spruce over portions of its range in the Northeast, and natural hybrids between white and black spruce have been reported in Minnesota (Little and Pauley, 1958). The relationship of white spruce to both black and red spruce, therefore, represents another interesting aspect of this taxonomic problem.

It was my objective to analyze this complex of spruce by examining two groups of chemicals: phenolic compounds in the foliage and monoterpenes in oleoresin taken from the cortex. This study was an attempt to assess the value of the application of chemical criteria to specific taxonomic problems in forest trees in general and spruces in particular. In focusing on two specific

chemical groups, phenolics and monoterpenes, it was also hoped that patterns of genetic differentiation in spruce would be further defined and that the evolutionary relationships between red, black and white spruce would become somewhat clearer.

The specific objectives of this study were:

1. To compare simple phenols and polyphenols in foliage of red, black, and white spruce.
2. To determine geographic variation in the phenolic patterns in red, black, and white spruce.
3. To determine physiological and environmental effects on phenolic patterns in spruce foliage, i.e.:
   (a) the effects of using dried versus fresh foliage.
   (b) the effects of season.
   (c) the effects of tissue age.
   (d) the effects of site.
4. To determine the qualitative and quantitative variation in monoterpenes of white and red spruce.
5. To compare quantitative monoterpene content between red, black, and white spruce.

CHAPTER I

PHENOLIC COMPOUNDS AS TAXONOMIC CRITERIA IN SPRUCE

Comparative phenolic chemistry has been used in systematic problems at several taxonomic levels (Alston and Turner, 1963a; Harborne, 1964 and 1967; and Swain, 1963 and 1966). The use of two-dimensional paper chromatographic techniques to reveal the patterns of phenolic compounds of species was pioneered and developed by Alston and Turner (1962 and 1963b). They were able to characterize species by the pattern of phenolic compounds on paper chromatograms. Not only could the relationships between species be detected by the degree of similarity of their patterns, but the method also proved highly valuable for the detection of interspecific hybridization.

Since that first work by Alston and Turner the application of phenolic compounds as taxonomic criteria has been used in the Pinaceae as well as in many other families. However, in the woody plants the greatest attention has been given to those compounds occurring in the wood and bark of the genus Pinus. Recently, Thielges (1969) has used paper chromatography of foliage

polyphenols to investigate the chemical relationships among species in the subsection Sylvestris of the genus Pinus. In addition, Hoff (1968) has found differences in the paper chromatographic patterns of the foliage polyphenols in Pinus monticola and P. flexilis that are useful in verifying hybrids between these two species.

In my study, paper chromatography of foliage phenolic compounds is used to determine the chemical relationships among white spruce (Picea glauca), black spruce (Picea mariana), and red spruce (Picea rubens). Samples were obtained from a number of populations representative of the geographic range of each species in the Northeast. The occurrence of hybridization in many sources of red and black spruce provided the opportunity to evaluate the use of phenolic compounds to study natural hybridization and introgression in forest trees.

Materials and Methods

Foliage samples for the chromatographic analysis of phenolic compounds were all obtained from trees 10- to 20-years-old planted in replicated seed source tests. The samples included 14 geographic origins of red spruce planted at Valcartier, Quebec and at Petawawa, Ontario; 12 origins of black spruce planted at Petawawa, Ontario; and 12 origins of white spruce planted at the W. K. Kellogg Forest in southern Michigan. The geographic locations of all sources sampled are presented in Figures 1, 2, and 3.

Figure 1.--Natural range of white spruce (Fowells, 1965) and location of sources analyzed for their phenolic constituents.

1658
1657
1659
1661
1655
1660
1649
1644
1662
1686
1676
MILES

Figure 2.--Natural range of black spruce (Fowells, 1965) and location of sources analyzed for their phenolic constituents.

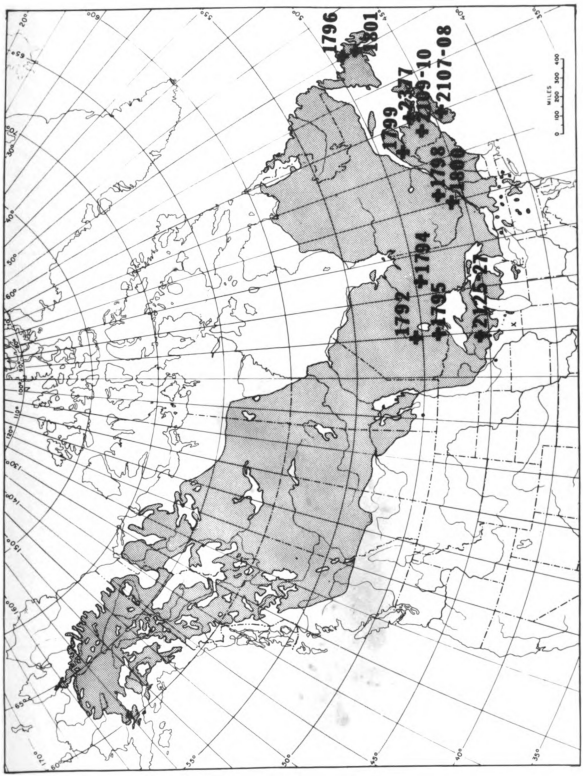
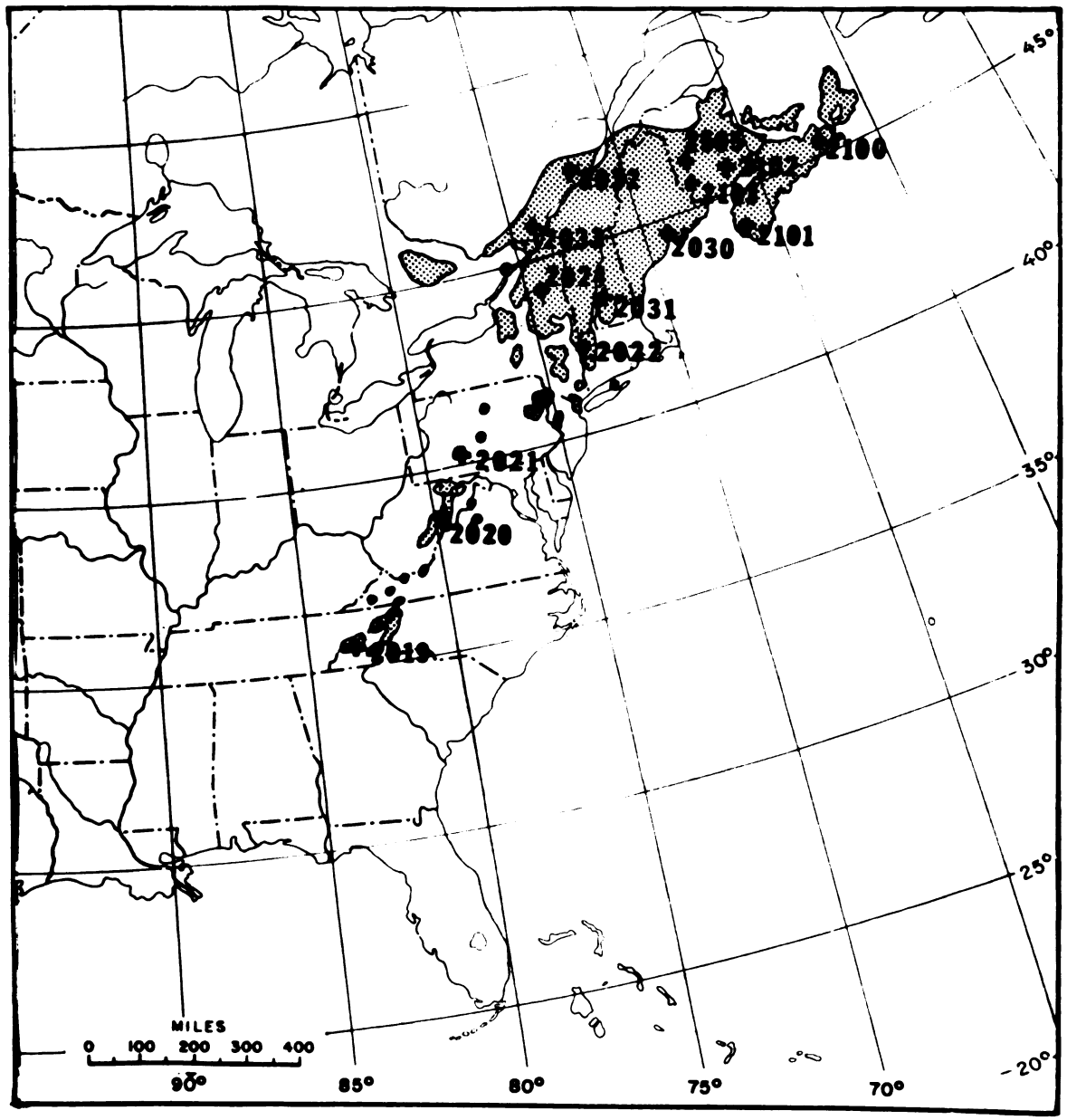

1796
1801
1799
1798
1794
1792
1795
2107-08
MILES

Figure 3.--Natural range of red spruce (Fowells, 1965) and location of sources analyzed for their phenolic constituents.


45°
40°
35°
30°
25°
20°
90°
85°
80°
75°
70°
MILES
0 100 200 300 400
2100
2030
101
2131
2022
2021
2020

To aid in the analysis of natural hybrids between red and black spruce, foliage samples were obtained from twelve known F_1 hybrids of these two species. These hybrids were obtained through controlled pollination and they are growing at the Petawawa Forest Experiment Station in Ontario, Canada.

Prior to making species comparisons, I selected foliage age, season of collection, and drying treatment for preliminary assessment of their effect on variation patterns in spruce phenolic compounds. Qualitative and quantitative variation was found between current-year, 1-year-old, and 2-year-old foliage; between samples collected in July and those collected in February; and between fresh and dry foliage. These differences are sufficiently large to necessitate their consideration in genetic studies using phenolic compounds, and standardized sampling techniques must be used if meaningful results are to be obtained.

The foliage samples of red and black spruce and their hybrids were collected in late June of 1968. White spruce foliage samples were collected in July of 1969. Foliage samples were all obtained from 1-year-old internodes of lateral branches distributed throughout the crown of the tree.

The foliage was oven-dried at 50° C until a constant dry weight was obtained. Two grams of dried foliage

from each of eight individual trees per geographic origin were bulked into two samples of four trees each for analysis. The 8-gram samples were extracted with 100 ml of boiling water for 2 minutes. The water and foliage was then homogenized for 2 minutes, filtered, and homogenized again with 100 ml of boiling water. This mixture was filtered and washed with 50 ml of boiling water. The filtrates were combined and transferred to a separatory funnel. The water extract was further extracted five times each with 50 ml portions of ethyl ether followed by n-butanol to yield two separate fractions of phenolic constituents. Phenols with only a few hydroxyl groups are soluble in ether, while butanol is a good general solvent for polyphenols. A 125-ml aliquot from the n-butanol extract was taken to near-dryness on a rotary film evaporator and brought up to 1 ml with n-butanol. The ether extract was evaporated under forced air, redissolved in ethanol, taken to near-dryness on a rotary film evaporator, and brought up to 1 ml with ethanol.

Phenols in both fractions were separated by two-dimensional descending chromatography. Fifty microliters of the extracts were applied with a micropipet to Whatman 3MM filter paper. The papers were irrigated in the first direction with either n-butanol, acetic acid, water (4:1:5) or benzene, acetic acid, water (6:7:3) for the butanol soluble and ether-soluble fractions, respectively.

Sodium formate, formic acid, water (10:1:200) was the solvent in the second direction for both fractions.

Dried chromatograms were examined under ultraviolet radiation and fumed with ammonia and reexamined in the ultraviolet. They were then sprayed with a solution of 1 gm of diazotized sulfanilic acid in 250 ml of water followed by 2N sodium hydroxide. In addition to noting their color reaction, the R_f values of each compound were measured, and those compounds which were found to be consistently present on these bases were numbered. A number of known substances were previously chromatographed separately and in combination with spruce extracts in order to provide marker compounds and also to tentatively identify a few of the spruce phenolics (Hanover and Wilkinson, 1969).

Results and Discussion

A total of 72 different compounds were found in the ether-soluble fractions of red, black, and white spruce. Another 90 compounds were found in the butanol-soluble fractions. However, individual chromatograms usually contained 15 to 35 compounds with excellent separation. Representative chromatograms for each species are illustrated in Figure 11 in the Appendix.

Individual compounds were characterized by their R_f values and color reactions which are given in Tables

23 and 24 of the Appendix. Composite chromatograms of the ether-soluble and butanol-soluble fractions are illustrated in Figure 12 of the Appendix.

Differences in black spruce, white spruce, and red spruce can be observed easily by visual examination of the chromatograms. White spruce is characterized by large amounts of each ether-soluble phenolic compound. On the basis of visual estimates of spot size and color intensity the individual simple phenolic compounds of white spruce appear to occur in concentrations 2 to 3 times greater than they occur in red and black spruce. Although these differences may be biochemical they could also be due to differences in needle shape and size and perhaps to differences in the ratios of surface area to volume. Pure red spruce is characterized by a scarcity of compounds in the ether fraction and the complete absence of compounds 4 and 40 in the ether-soluble and butanol-soluble fractions, respectively.

Many of the phenolic compounds occurred in every sample analyzed and showed little quantitative variation. Forty-nine compounds do show patterns of variation associated with species. These distinctive compounds are of taxonomic interest. Their frequencies of occurrence in each species are presented in Tables 1 and 2.

The most valuable compounds from a taxonomic point of view are those which are always present in one species

Table 1.--Frequency of occurrence of the distinctive ether-soluble phenolic compounds in foliage of white, black and red spruce.

Compound Number	Species		
	White Spruce	Black Spruce	Red Spruce
	----------Percent of Samples----------		
4	100	100	62
8	00	18	69
10	83	45	19
11	00	91	27
12	41	09	69
13	100	36	04
14	46	100	92
17	00	00	31
19	50	14	08
26	00	50	31
31	96	05	00
32	92	45	81
35	62	05	00
36	100	00	00
37	50	05	00
46	33	86	00
47	00	55	00
58	92	100	62

Table 2.--Frequency of occurrence of the distinctive butanol-soluble phenolic compounds in foliage of white, black, and red spruce.

Compound Number	Species		
	White Spruce	Black Spruce	Red Spruce
	----------Percent of Samples----------		
2	100	100	31
3	00	14	96
7	76	00	00
8	04	96	50
10	92	100	42
11	21	45	00
12	63	42	23
13	84	00	00
15	00	00	65
22	04	32	96
29	100	32	08
33	21	00	88
35	25	00	04
37	00	00	46
40	100	100	65
43	00	00	50
45	79	54	96
48	84	32	88
53	83	04	31
54	29	04	36
55	96	54	04
59	00	00	54
61	50	82	04
71	04	32	36
73	00	00	42
74	67	28	100
76	00	00	42
77	00	00	42
78	04	16	50
82	08	00	72
84	00	00	42

but do not occur in another species. On this basis only compound 36 in the ether-soluble fraction is suitable for separating all sources of white spruce from both black and red spruce. Twenty-five compounds are completely absent in one or two of the three species, but they occur in less than 100 percent frequency in the species in which they are present. These compounds are suitable for the separation of spruce species only if large numbers of samples are obtained and separation is based on frequency of occurrence.

However, the full taxonomic potential of many of these compounds is not indicated in Tables 1 and 2 due to the diversity of sources sampled. Many of the sources of both red and black spruce have been affected by hybridization. For example, compounds 4 and 40 in the ether-soluble and butanol-soluble fractions, respectively, are the most useful for separating red and black spruce, but the hybridization occurring in many of the sources of red spruce accounts for their presence in 62 percent (ether-soluble) and 65 percent (butanol-soluble) of the samples analyzed. A similar situation occurs for many of the other compounds listed in Tables 1 and 2.

To determine the total chemical similarity between species each compound was considered as a separate character and the data were quantified by determing the percent of compounds in common using the formula:

$$\frac{\text{Compounds in common for species A + B}}{\text{Total compounds in A + B}} \times 100$$

The percentages of compounds in common between white, black, and red spruce are presented in Table 3. The compounds in common within species were calculated as the mean value of the chemical similarities between all geographic sources sampled. As expected the intraspecific chemical affinity values are the highest. Deviation from 100 percent within species represents geographic and individual tree variation. Of the three species, white and black spruce are the most similar in their phenolic composition. Red spruce is more similar to black spruce than to white spruce. The closer phylogenetic relationship between black spruce and red spruce than that of either species to white spruce as proposed by Wright (1955) is not upheld by examination of their phenolic constituents. Although no specific adaptive function has been proposed for phenolic compounds in spruce, perhaps some of these compounds have been subject to climatic or other selective factors. The greater similarity in the phenolic compounds of black and white spruce is congruous with their similarity in habitat, as both are indigenous to boreal regions while red spruce is native to cool-temperate climates.

Table 3.--Degree of similarity of the phenolic compounds of two fractions in foliage of white, black, and red spruce.

	Species					
	White Spruce		Black Spruce		Red Spruce	
	-----Percent of Compounds in Common[1]----					
White Spruce	91	88	--	--	--	--
Black Spruce	78	77	88	84	--	--
Red Spruce	71	65	76	69	83	85

[1]The First of the two values represents percent of compounds in common in the ether-soluble fraction, the second represents the butanol-soluble fraction.

Although differences in the phenolic composition of these three species occurs, the similarity between them is much greater than between species of pine in the subsection Sylvestres (Thielges, 1969). This supports the contention of Wright (1955) that the genus Picea is relatively homogeneous, and his comparison of the genus to a single subsection of Pinus in morphological variability appears to apply to phenolic constituents as well.

Geographic Variation

Determining the geographic variation patterns for a taxonomic trait is imperative. Many chemotaxonomic studies in the past have assumed species uniformity rather than assess intraspecific variation. Determination of the

geographic variation patterns in the phenolic compounds of spruce also may be important in characterizing evolutionary patterns within a species.

Variability in phenolic compounds was encountered between geographic sources and between individual samples of the same source. Chemical similarity between sources and between replicates for each species are presented in Table 4. In both the ether- and butanol-soluble fractions greater variation in phenolic compounds occurs between replicates than between geographic sources. Apparently a large amount of individual tree variation occurs in the phenolic compounds of spruce.

Table 4.--Chemical similarity between replicates and between geographic sources in red, black, and white spruce.

Species	Ether Fraction		Butanol Fraction	
	Replicates	Sources	Replicates	Sources
	-----Percent of Compounds in Common------			
Red Spruce	80	83	82	86
Black Spruce	87	88	83	85
White Spruce	90	91	87	88

Although the effect of planting site was not tested rigorously, replicate samples of red spruce foliage were obtained from two widely separated plantations while replicate foliage samples of white and black spruce were obtained from the same plantation. Examination of the chemical similarities in Table 4 shows that the differences between geographic sources and replicates are higher in red spruce than in black or white spruce. From the limited data it cannot be determined whether this effect is genetic or environmental. This can only be determined from clonal material planted in diverse locations. However, in the only study of this nature McClure and Alston (1964) reported that genetically identical plants of Spirodela oligorhiza grown under 52 different environmental treatments exhibited little qualitative difference in their phenolic compounds.

Geographic sources of each species were compared separately by determining the percent of compounds in common between each source. Chemical similarity between sources for red, black, and white spruce are presented in Tables 25, 26, and 27 of the Appendix. In red and black spruce the most notable feature is the low chemical affinity between sources in which hybridization has occurred and sources of the pure species. The sources of red spruce from North Carolina and West Virginia, for example, are very similar in both their ether-soluble and butanol-soluble

compounds. The chemical affinities between these two sources are 92 and 91 percent for the ether and butanol fractions, respectively.

On the other hand, chemical affinities are much lower between these two sources and source 2033 from Quebec in which extensive hybridization with black spruce has been shown by Morgenstern and Farrar (1964). The chemical affinities between the North Carolina source and source 2033 from Quebec are 75 and 78 percent for the ether-soluble and butanol-soluble fractions, respectively. The affinities between the West Virginia source and source 2033 are 74 percent in both fractions. In red spruce and to some extent in black spruce geographic variation is largely associated with hybridization.

The chemical similarity between geographic sources of white spruce is greater than in red or black spruce. Variation of the phenolic compounds in white spruce shows no geographic patterns in that adjacent sources in many cases are less similar than widely separated sources.

Analysis of Hybridization in Red and Black Spruce

Most of the sources of red and black spruce which were sampled for comparison of their phenolic constituents were previously examined by Morgenstern and Farrar (1964) by character association analyses using morphological

traits. Their findings indicate that red and black spruce are two species that were geographically isolated in the past but have come in contact since the Pleistocene and are hybridizing. They also concluded that nearly all populations in the overlapping areas of the ranges of these two species have been affected by introgressive hybridization.

Phenolic compounds have recently been used to detect hybridization in several spruce species. On the basis of two phenolic compounds found in the foliage of white spruce but not in Sitka spruce (Picea sitchensis), Hanover and Wilkinson (1969) were able to show introgressive hybridization between these two species in the Skeena River area of British Columbia. The interpretation of hybridization on the basis of phenolic constituents was supported by similar conclusions reached by Daubenmire (1968) who studied the morphology of cones, twigs, and needles of the same populations. LaRoi and Dugle (1968) used phenolic compounds to study the white and Engelmann spruce (Picea engelmannii) complex of the northwest United States and Canada. They did not find any species-specific compounds which distinguish white from Engelmann spruce.

In order to study the natural hybridization in red and black spruce, I used populations of each species growing in isolation of the other species as standards.

Red spruce from North Carolina, source 2019, was considered to be representative of the pure species. Black spruce from Wisconsin, sources 2125-27, was considered to be pure black spruce. Morgenstern and Farrar (1964) also based their pure grades of red spruce on source 2019, but they based their pure grades of black spruce on northern Ontario sources. Foliage from 20 pure red spruce, source 2019, 12 pure black spruce, sources 2125-27, and the 12 F_1 hybrids was extracted and chromatographed as previously described except that the samples were not bulked. The phenolic compounds in the samples of red and black spruce served as a basis for examination of the phenolic compounds in the F_1 hybrids and for the detection of hybridization in natural populations.

Black spruce and pure red spruce had 66 percent of their simple phenols and 64 percent of their polyphenols in common. On the other hand, black spruce and all sources of red spruce had 76 and 69 percent of their compounds in common (Table 3). Thirty-one phenolic compounds of pure red spruce and pure black spruce are species-specific. Distinctive phenolic compounds of red and black spruce and F_1 hybrids occurring in the ether-soluble and butanol-soluble fractions, respectively, are presented in Tables 5 and 6. Compounds 4 and 11 in the ether fraction and compounds 2, 37, and 40 in the butanol fraction are specific to black spruce and occurred in every sample analyzed.

Table 5.--Frequency of occurrence of the distinctive ether-soluble compounds in foliage of red spruce, black spruce, and F_1 hybrids.

Compound Number	Species		
	Red Spruce	Black Spruce	F_1 Hybrids
	----------Percent of Samples----------		
2	20	66	17
4	00	100	100
8	50	08	42
10	00	75	00
11	00	100	100
13	10	75	100
19	40	33	08
20	40	85	33
21	25	00	17
25	10	33	42
26	70	92	25
38	20	85	85
39	100	100	50
42	00	75	17
43	00	58	00
46	10	75	00
50	00	17	08
53	20	00	33
58	00	50	00
61	00	00	17
63	00	00	17
64	00	00	17
65	00	00	25

Table 6.--Frequency of occurrence of the distinctive butanol-soluble compounds in foliage of red spruce, black spruce, and F_1 hybrids.

Compound Number	Species		
	Red Spruce	Black Spruce	F_1 Hybrids
	----------Percent of Samples----------		
1	40	92	100
2	00	100	100
3	60	00	09
6	30	92	100
8	90	08	63
10	00	92	91
11	00	25	55
12	00	58	91
15	50	00	09
16	100	25	100
26	00	66	18
33	100	00	27
35	50	25	27
37	00	100	100
39	40	08	55
40	00	100	100
43	00	50	27
47	00	33	09
50	00	00	50
55	00	83	63
58	00	08	27
59	100	00	00
61	00	66	63
67	00	00	18
72	80	00	00
73	30	00	55
74	100	00	91
76	80	00	09
80	50	00	09
82	70	00	27
83	00	50	00
84	70	00	09

Compounds 33, 59, and 74 were found in every red spruce sampled but not in black spruce. Nine other compounds were found only in red spruce and fifteen other compounds were found only in black spruce, but these compounds did not occur in all of the samples.

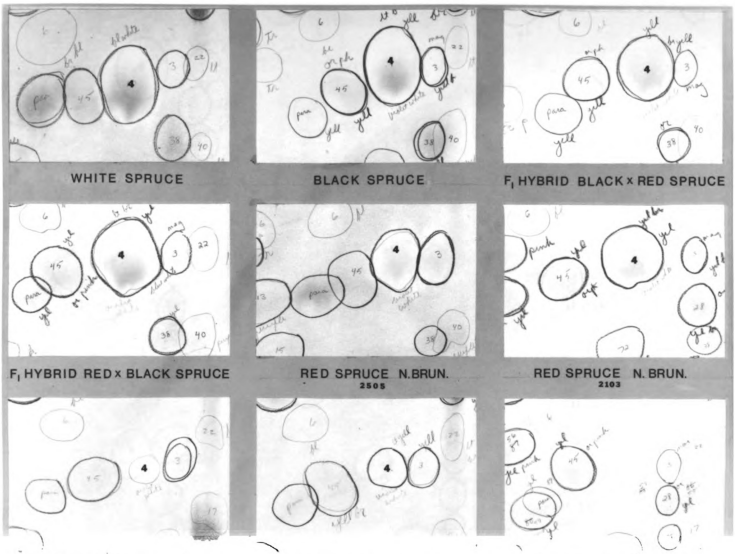
The F_1 hybrids had 79 and 72 percent of their ether-soluble and butanol-soluble compounds, respectively, in common with black spruce. They also had 74 percent of their compounds from both fractions in common with pure red spruce. Compounds 4 and 11 in the ether fraction, and 2, 37, and 40 in the butanol fraction which occur only in black spruce, were found in all of the F_1 hybrids. Moreover, these compounds did not vary quantitatively from the amounts found in pure black spruce. Although the gene control of these compounds cannot be determined without analysis of the parents of these hybrids it appears that these compounds may be dominant. Dominance with respect to the inheritance of phenolics has been found to be the usual case in interspecific hybrids of Baptisia (Alston and Turner, 1962; and Alston and Hempel, 1964). Six compounds were found only in the F_1 hybrids. These compounds may be hybrid compounds but this cannot be determined without analysis of the actual parents. However, hybrid compounds have often been found in interspecific hybrids of the genus Baptisia (Alston, et al., 1965).

Compounds 4 and 40 in the ether and butanol fractions, respectively, are the most useful for detecting hybridization in natural populations of red spruce. However, these compounds are not suitable for detecting introgression following hybridization in black spruce because they occur in equal and large concentrations in every black spruce sampled. However, visual quantitative estimates of the concentrations of these compounds can be used for purposes of detecting the degree of hybridization in red spruce. These same compounds were utilized by Hanover and Wilkinson (1969) for detecting hybridization in white and Sitka spruce.

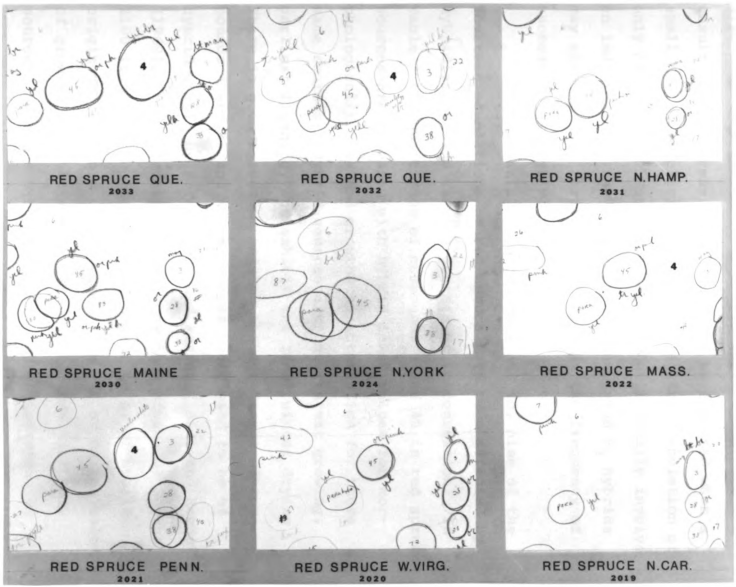
The concentrations of both of these compounds vary continuously from complete absence to near that of black spruce in the various sources of red spruce. The quantitative variation of compound 4 in geographic sources of red spruce is presented in Figure 4. Compound 40 varies in a similar manner, but it is visible only under ultraviolet radiation and is obscured by other compounds after application of the spray reagents.

The variation in concentrations of these two compounds suggests that introgression or crossing back to a parent species, in this case red spruce, has followed hybridization as the F_1 hybrids contain these compounds in concentrations equal to those in black spruce. Although Morgenstern and Farrar (1964) encountered some intermediates

Figure 4a and 4b.--Chromatographic evidence of quantitative variation in compound 4 in geographic sources of red spruce. Only one section of the two-dimensional chromatograms is illustrated and chromatogram sections of white and black spruce and F_1 hybrids are included for comparison.


WHITE SPRUCE
BLACK SPRUCE
F1 HYBRID BLACK x RED SPRUCE
F1 HYBRID RED x BLACK SPRUCE
RED SPRUCE N.BRUN.
2505
RED SPRUCE N. BRUN.
2103
RED SPRUCE N. BRUN.
2102
RED SPRUCE N.SCOTIA
2101
RED SPRUCE N.SCOTIA
2100

(a)


RED SPRUCE QUE.
2033
RED SPRUCE QUE.
2032
RED SPRUCE N.HAMP.
2031
RED SPRUCE MAINE
2030
RED SPRUCE N.YORK
2024
RED SPRUCE MASS.
2022
RED SPRUCE PENN.
2021
RED SPRUCE W.VIRG.
2020
RED SPRUCE N.CAR.
2019

(b)

between red and black spruce in natural populations, they also concluded that most of the variable types were the result of introgression. This would be expected when a small hybrid population is close to a large population of only one parent. Subsequent crosses will usually involve an individual of the parent population. The F_1 hybrids may also be less fertile which reduces the frequency of successful matings with them.

On the basis of compounds 4 and 40, nine of the fourteen populations of red spruce have been affected by hybridization and varying degrees of introgression. In Table 7 the quantities of compounds 4 and 40 in red spruce sources are compared with hybrid index values for morphological traits and growth characteristics for these same sources. Hybrid sources are the fastest growing, earliest flushing, and exhibit the least winter drying. There is good agreement between the occurrence of compounds 4 and 40 and those sources postulated to be of hybrid origin by Morgenstern and Farrar (1964) and Roche (1969). Some discrepancies are evident however. Source 2100 from Nova Scotia does not appear to be of hybrid origin on the basis of compounds 4 and 40 or on the basis of growth characteristics. The hybrid index for this source suggests a hybrid origin. These discrepancies are probably due to differences in sampling schemes. The same individuals were not sampled in the three separate

Table 7.--Concentration of compounds 4 and 40, hybrid indices, and growth characteristics in 14 geographic sources of red spruce.

Source	Compound				Hybrid Index[1]	Growth Characters (Roche, 1969)		
	4		40			Height at Age 14	Date of Flushing	Winter Drying
	Rep. 1	Rep. 2	Rep. 1	Rep. 2				
	Percent of Amount in Black Spruce					Meters	Days after June 1	Percent of Trees
2019-NC	0	0	0	0	21-26	1.09	21.5	96
2020-WV	0	0	0	0	24-26	.71	26.5	78
2021-PE	60	60	60	60	16-26	1.27	21.0	86
2022-MS	20	40	20	20	17-26	.94	24.0	80
2024-NY	0	0	0	0	21-26	.98	24.0	80
2030-MA	0	20	0	20	17-26	.94	25.5	78
2031-NH	0	0	0	0	20-26	1.03	20.0	82
2032-QE	40	60	20	20	19-26	1.18	16.5	86
2033-QE	80	60	60	40	2-26	1.45	12.5	36
2100-NS	0	0	0	0	17-26	.98	22.5	90
2101-NS	20	40	20	20	14-26	1.17	18.0	82
2102-NB	20	20	20	20	15-26	.89	25.0	78
2103-NB	80	60	60	40	16-26	1.20	17.0	70
2505-NB	20	80	40	40	6-26	1.46	16.0	56

[1]Obtained from Morgenstern and Farrar (1964). Pure black spruce includes the index values 0-2, perfect intermediates 12-14, and pure red spruce 24-26.

studies, and many of the individual trees in each source may be of the pure species.

Many compounds other than 4 and 40 are of diagnostic value for determining the degree of hybridization of black and red spruce. By determining the number of their compounds in common each geographic source of red spruce and black spruce was compared with pure red spruce from North Carolina and pure black spruce from Wisconsin. The chemical affinities for the ether-soluble and butanol-soluble fractions, respectively, are presented in the form of scatter diagrams in Figures 5 and 6. Affinity values for the F_1 hybrids with pure black spruce and pure red spruce are included for comparison. Although intermediacy for the F_1 hybrids is evident, the affinities with both species are somewhat low due to the presence of hybrid compounds.

The ether-soluble fraction is not suitable for detecting hybridization in black spruce. All sources show a similar high affinity with black spruce from Wisconsin and low affinity for pure red spruce. On the other hand, a number of red spruce sources are more similar to black spruce in their ether-soluble constituents than they are to red spruce. On the basis of their total ether-soluble phenolic constituents, sources 2505, 2033, 2100, 2022, 2103, and 2021 appear to be of hybrid origin. Sources 2101, 2030, and possibly 2102 are nearly equal in their

Figure 5.--Species affinity in ether-soluble phenolics for geographic sources of red and black spruce.

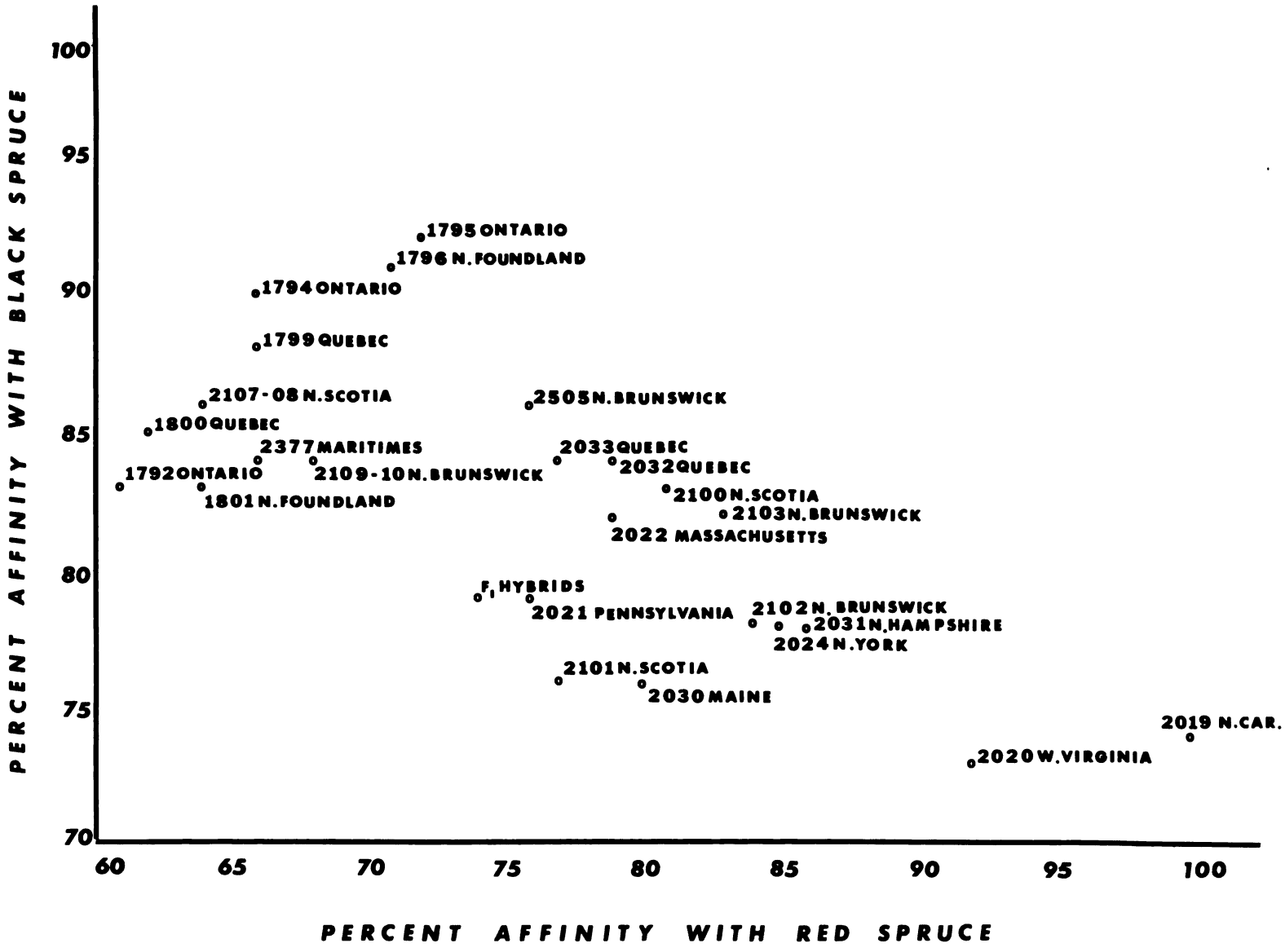
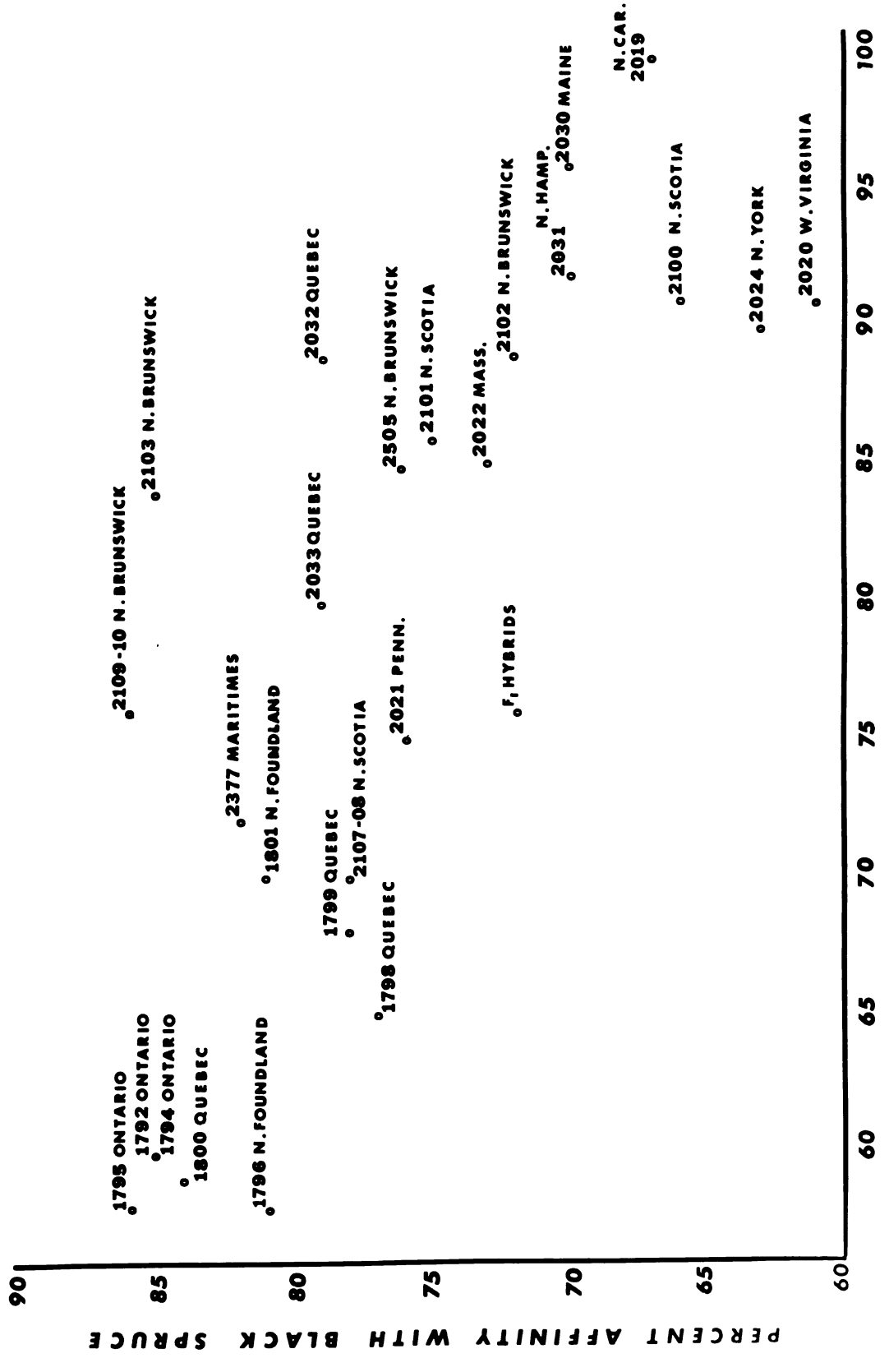

PERCENT AFFINITY WITH BLACK SPRUCE
100
95
90
85
80
75
70
60
65
70
75
80
85
90
95
100
PERCENT AFFINITY WITH RED SPRUCE
1795 ONTARIO
1796 N. FOUNDLAND
1794 ONTARIO
1799 QUEBEC
2107-08 N.SCOTIA
2505 N. BRUNSWICK
1800 QUEBEC
2377 MARITIMES
2033 QUEBEC
2032 QUEBEC
1792 ONTARIO
2109-10 N. BRUNSWICK
2100 N.SCOTIA
1801 N. FOUNDLAND
2103 N. BRUNSWICK
2022 MASSACHUSETTS
F1 HYBRIDS
2021 PENNSYLVANIA
2102 N. BRUNSWICK
2031 N. HAMPSHIRE
2024 N. YORK
2101 N.SCOTIA
2030 MAINE
2019 N.CAR.
2020 W. VIRGINIA

Figure 6.--Species affinity in butanol-soluble phenolics for geographic sources of red and black spruce.


PERCENT AFFINITY WITH BLACK SPRUCE
PERCENT AFFINITY WITH RED SPRUCE
90
85
80
75
70
65
60
60
65
70
75
80
85
90
95
100
1795 ONTARIO
1792 ONTARIO
1794 ONTARIO
1800 QUEBEC
1796 N.FOUNDLAND
2377 MARITIMES
1801 N.FOUNDLAND
2109-10 N.BRUNSWICK
2103 N.BRUNSWICK
1799 QUEBEC
2107-08 N.SCOTIA
1798 QUEBEC
2021 PENN.
2033 QUEBEC
2032 QUEBEC
2505 N.BRUNSWICK
2101 N.SCOTIA
2022 MASS.
F1 HYBRIDS
2102 N.BRUNSWICK
N.HAMP.
2031
2030 MAINE
N.CAR.
2019
2100 N.SCOTIA
2024 N.YORK
2020 W.VIRGINIA

affinity with each species and as indicated previously they are probably also of hybrid origin. Even sources 2024 and 2031 are closer to black spruce in their ether-soluble phenolics than are the two Southern Appalachian sources.

The butanol-soluble phenolics are suitable for detecting hybridization in both red and black spruce. Black spruce sources 1798, 1799, 1801, 2377, 2107-08, and 2109-10 resemble red spruce in butanol-soluble compounds to a much greater extent than the sources from Ontario. All of these sources originate in areas where red spruce is present.

Red spruce sources 2021, 2033, 2103, 2505, 2032, 2101, 2022, and 2102 again appear to be of hybrid origin as indicated by their intermediate position on the scatter diagram (Figure 6).

Comparison of chemical affinities with hybrid indices and growth characters for red spruce in Table 8 reveals a general correspondence between the chemical and growth data. Low affinities with pure red spruce or high affinities with black spruce suggests hybridization. The populations indicated to be of hybrid origin on the basis of their phenolic constituents correspond to those indicated to be of hybrid origin on a morphological basis.

There is also a correspondence of chemical affinity values for geographic sources of black spruce with

Table 8.--Species affinity values, hybrid indices for morphological traits, and growth characteristics in 14 geographic sources of red spruce.

Source	Red Spruce-N. Car.		Black Spruce-Wisc.		Hybrid Index[1]	Growth Characters (Roche, 1969)		
	Ether Fraction	Butanol Fraction	Ether Fraction	Butanol Fraction		Height at Age 14	Date of Flushing	Winter Drying
	---Percent of Compounds in Common-----					Meters	Days after June 1	Percent of Trees
2019-NC	100	100	74	67	21-26	1.09	21.5	96
2020-WV	92	91	73	61	24-26	.71	26.5	78
2021-PE	76	75	79	76	16-26	1.27	21.0	86
2022-MS	79	85	82	71	17-26	.94	24.0	80
2024-NY	85	90	78	63	21-26	.98	24.0	80
2030-MA	80	96	76	70	17-26	.94	25.5	78
2031-NH	86	92	78	70	20-26	1.03	20.0	82
2032-QE	79	89	84	79	19-26	1.17	16.5	86
2033-QE	78	79	84	79	2-26	1.45	12.5	36
2100-NS	81	91	83	66	17-26	.98	22.5	90
2101-NS	77	86	76	75	14-26	1.17	18.0	82
2102-NB	84	89	78	72	15-26	.89	25.0	78
2103-NB	83	84	82	85	16-26	1.20	17.0	70
2505-NB	76	85	86	76	6-26	1.46	16.0	56

[1]Obtained from Morgenstern and Farrar (1964). Pure black spruce includes the index values 0-2, perfect intermediates 12-14, and pure red spruce the values 24-26.

hybrid index values (Table 9). However, phenolic constituents do not indicate that source 1800 from Quebec is of hybrid origin. Conversely, source 1801 from Newfoundland appears to be of hybrid origin on the basis of its phenolic constituents, but not on the basis of morphology. As already mentioned these discrepancies may be entirely due to sampling procedures.

It was not considered necessary to identify the compounds for the purpose of comparing species of spruce and their hybrids (Alston and Turner, 1963a). However, when the taxonomic significance of compounds 4 and 40 is considered, it would be desirable to know their identity so that interpretation of their distribution in spruce species may be made.

Compound 40 was tentatively identified as the glucoside, pungenin, by Hanover and Wilkinson (1969). Neish (1957) first isolated pungenin from blue spruce (Picea pungens). I found compound 40 to vary in concentration with season; in both black and white spruce compound 40 occurred in low concentrations in the winter. Neish (1958), on the other hand, found pungenin in white spruce to be lower in concentration in the summer. This suggests that the identification is questionable. In addition, Rast, et al. (1964) did not find pungenin in samples of black spruce while compound 40 was present in every black spruce which I sampled. Obviously, further

Table 9.--Species affinities in butanol-soluble phenolics and hybrid indices for morphological traits in geographic sources of black spruce.

Source	Black Spruce-Wisc.	Red Spruce-N. Car.	Hybrid Index[1]
	---Percent of Compound in Common----		
1792-ON	84	59	0-3
1794-ON	85	60	0-3
1795-ON	86	58	0-3
1800-QE	84	59	0-11
1799-QE	78	68	0-7
1798-QE	77	65	0-8
1801-NF	81	70	0-2
2107-08-NS	78	70	0-22
			0-19
2109-10-NB	86	76	0-13
			0-16
1796-NF	81	58	----
2377-MT	82	72	----

[1]Obtained from Morgenstern and Farrar, 1964. Pure black spruce includes the index values 0-2, perfect intermediates 12-14, and pure red spruce 24-26.

steps are needed for positive identification of this constituent.

It was not possible to identify compound 4, even tentatively, by color reaction or R_f values. However, the reactions of compound 4 to several spray reagents suggests that it may belong to the catechin-phloroglucinol-resorcinol group of phenols (Hergert, 1960). Compound 4 also reacted to a sodium nitrite spray reagent in a manner characteristic of chlorogenic acid, but many of its other properties do not substantiate this possibility.

CHAPTER II

GENETIC VARIATION IN THE MONOTERPENE COMPOSITION OF WHITE SPRUCE (*PICEA GLAUCA*)

Terpenoid compounds have been used in the past for comparative biochemical purposes and for the verification of hybridity. With the advent of gas-liquid chromatography for rapid separation of terpene compounds and increased use of computers for numerical analysis, the study of quantitative as well as qualitative variation in terpene levels within a species has become considerably more feasible.

Monoterpene composition of cortical oleoresin has been shown to be under strong genetic control in pine (Hanover, 1966a and 1966b; Hilton, 1968; Squillace and Fisher, 1966; and Tobolski, 1968). Since the monoterpenes vary only slightly with the environment they satisfy the main requirement of a taxonomic tool and should be valuable markers for population genetic studies in *Pinus* and other oleoresin-producing genera such as *Picea*. Studies of qualitative and quantitative variation in monoterpene composition can contribute to the knowledge of genetic

differentiation within a species and may give some insight into evolutionary patterns when considered along with other criteria.

Determination of chemical variation patterns in species of Picea can be especially important since the basic spruce type has changed relatively little in morphological characters while becoming differentiated into the presently recognized species (Wright, 1955). White spruce (Picea glauca) occupies an extensive range across boreal Canada (Figure 7). Over such a wide range geographic and other barriers to crossing are sure to occur and geographic variation in terpene composition is expected.

Geographic variation in cortical monoterpenes has been reported in several species of Pinus. Hilton (1968); Squillace and Fisher (1966); and Tobolski (1968) reported on geographic variation in cortical monoterpenes. Geographic variation in monoterpenes was also shown in three populations of Douglas-fir (Pseudotsuga menziesii) in Montana and Idaho (Hanover and Furniss, 1966). Von Rudloff (1967) examined the leaf oil of eastern and western sources of white spruce. My study was undertaken to assess the variability and genetic differentiation of cortical monoterpenes in white spruce.

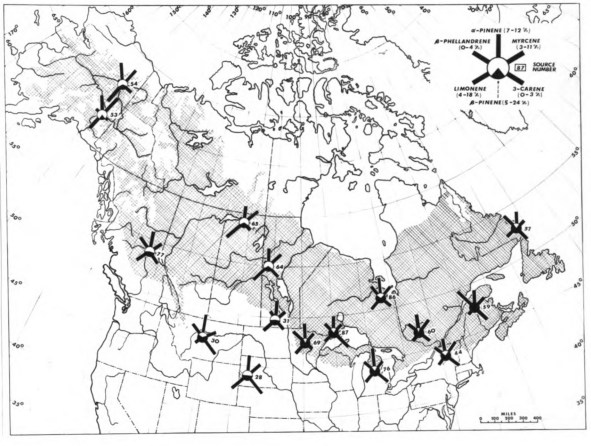


Figure 7.--Natural range of white spruce (Picea glauca) (Fowells, 1965), location of seed sources, and geographic variation in the principal monoterpenes. The mean amount of each terpene is represented by the length of bar or degree of shading of the circle. Sources are designated by the last two digits of the number assigned by the Northern Institute of Forest Genetics and each should be preceded by 16.

Materials and Methods

Cortical oleoresin samples were collected from 16 geographic origins of white spruce (Figure 7) planted in a replicated growth test at W. K. Kellogg Forest near Augusta, Michigan. This plantation was established in 1963 with 2-3 stock and was 10 years old at the time of sampling. Two trees were sampled from each of five replicates for a total of ten trees per source.

Samples of oleoresin were taken over a 2-month period from July 1968 to September 1968. Small incisions were made into the phloem of the 1-year-old internode of the main stem. Ten µl samples of the exuded oleoresin were immediately drawn into calibrated 50 µl capillary tubes. The capillary tubes containing the samples were placed in sealed centrifuge tubes and refrigerated until analysis.

All samples were analyzed quantitatively by gas-liquid chromatography on the day following collection. At the time of analysis each sample was diluted with 30 µl of acetone. A 2 µl aliquot from this solution was injected into a F and M model 700 gas chromatograph equipped with a flame ionization detector and Disc integrator. The chromatograph column was stainless steel, 1/4 inch in diameter and 8 feet long. The column was packed with chromosorb W-AW coated with 10% polypropylene glycol.

Column temperature was 117°C, detector 200°C, injection port 190°C, and the helium flow rate was 130 ml/min.

The monoterpenes were identified by comparing relative retention times of the unknowns with those of known compounds. Quantitative determinations based on peak area integrator values were derived from standard curves prepared from known terpene concentrations run on the same column and under the same conditions as the unknown samples. Monoterpene composition is expressed as a percentage of the oleoresin, and the total terpene content was obtained by summing all values for the individual terpene concentrations except those of camphene and α-terpinene.

An analysis of variance was performed on each set of data. Degrees of freedom were 15, 4 and 60 for origin, replicate and error respectively. Duncan's new multiple range test was used for making comparisons between source means. In addition a cluster analysis described by Flake and Turner (1968) was used to examine patterns of variation associated with geographic origin.

Results and Discussion

Cortical oleoresin of white spruce contained nine detectable monoterpenes. Limonene, α- and β-pinene, and myrcene were present in the largest amounts (Table 10). This is in agreement with the stem oil analysis of white

Table 10.--Cortical monoterpene concentration in 16 geographic origins of white spruce.

Geographic Origin	Monoterpene						
	α-Pinene	β-Pinene	Myrcene	3-Carene	Limonene	β-Phellandrene	Total Monoterpene
	Percent of Oleoresin						
1654-AL	10.2	8.6	7.4	0.8	15.4	0.1	42.5
1653-AL	8.6	4.7	10.5	0.3	12.3	0.6	37.5
1677-BC	10.2	11.0	4.8	1.2	7.4	2.8	37.5
1630-MO	11.8	15.6	4.4	2.2	3.7	2.3	40.3
1665-SA	7.5	8.0	7.8	0.6	17.8	0.2	42.0
1628-SD	9.7	11.9	6.8	2.7	13.6	0.1	45.0
1664-MA	8.3	7.6	7.1	0.9	14.6	0.2	38.7
1631-MA	9.4	11.2	4.6	1.1	10.1	1.0	37.4
1669-MN	8.3	20.4	3.6	0.6	7.0	2.3	42.2
1687-ON	7.6	19.2	3.9	2.5	6.3	1.6	41.2
1676-MI	10.3	19.4	3.2	1.5	7.0	1.6	43.1
1686-ON	8.7	17.0	4.6	0.9	7.6	2.0	40.9
1660-QE	8.4	18.1	6.6	1.2	4.9	1.8	41.1
1644-NY	7.7	21.5	6.8	1.7	5.5	1.9	45.1
1659-NB	8.2	23.8	4.9	1.8	3.6	4.1	46.5
1657-LB	7.6	19.2	3.4	1.5	7.0	2.7	41.6

spruce by von Schantz and Juvonen (1966). Camphene occurred in small quantities in all trees, and α-terpinine occurred in small quantities in some trees; both were omitted from the statistical analysis. Terpinolene was also present in very small amounts and is included in the analysis only because of the consistent positive correlation between this terpene and 3-carene. The remaining two terpenes, 3-carene and β-phellandrene, were variable; concentrations ranged from complete absence to about 10 percent of the oleoresin in individual trees (Table 11).

The concentration of β-pinene, limonene, β-phellandrene and 3-carene differed significantly among geographic origin of the seed source and exhibited a distinct geographic pattern (Tables 10 and 11, Figure 7). On the other hand individual tree variation in the other two major terpenes of white spruce, α-pinene and myrcene, was large in comparison to variation between sources and is indicative of a lack of genetic differentiation of these terpenes over the widely separated geographic origins represented in this study.

The concentration of β-pinene was consistently lower in western than in eastern sources (Table 12). Conversely, limonene was present in the lowest concentrations in the eastern sources. Variation in β-phellandrene and 3-carene concentration also approximates an east-west pattern with both of these terpenes being lowest in

Table 11.--Ranges of cortical monoterpene concentration in 16 geographic origins of white spruce.

Geographic Origin	Monoterpene					
	α-Pinene	β-Pinene	Myrcene	3-Carene	Limonene	β-Phellandrene
	Percent of Oleoresin					
1654-AL	2-18	1-20	3-13	0-3	11-21	0-1
1653-AL	3-21	1-9	3-17	0-1	8-18	0-4
1677-BC	4-15	9-29	3-12	0-3	1-14	0-3
1630-MO	8-15	10-23	1-14	0-8	0-7	0-7
1665-SA	5-11	1-16	3-16	0-3	8-27	0-1
1628-SD	5-13	6-18	2-29	2-4	10-20	0-0
1664-MA	4-13	1-13	2-12	0-3	3-21	0-1
1631-MA	4-14	3-19	2-10	0-5	5-17	0-2
1669-MN	6-10	16-25	1-11	0-1	3-11	1-4
1687-ON	6-9	8-28	2-15	1-6	2-9	0-3
1676-MI	7-16	11-27	2-8	0-4	3-11	0-3
1686-ON	5-16	10-26	2-11	0-4	5-10	0-8
1660-QE	4-13	9-25	3-14	0-4	2-8	1-4
1644-NY	6-10	12-29	3-11	1-5	1-10	0-3
1659-NB	6-11	15-31	3-11	0-4	2-8	2-11
1657-LB	5-11	9-27	2-10	0-3	2-12	0-8

Table 12.--Ranking of white spruce sources by percentages of beta-pinene and limonene.[1]

Beta-pinene		Limonene	
Source	Percent of Oleoresin	Source	Percent of Oleoresin
59-NB	23.8	65-SASK	17.8
44-NY	21.5	54-ALAS	15.4
69-MIN	20.4	64-MAN	14.6
76-MICH	19.4	28-SDAK	13.6
57-LAB	19.2	53-ALAS	12.3
87-Ont.	19.2	31-MAN	10.1
60-QUE	18.1	86-ONT	7.6
86-ONT	17.0	77-BC	7.4
30-MONT	15.6	76-MICH	7.0
28-SDAK	11.9	69-MINN	7.0
31-MAN	11.2	57-LAB	7.0
77-BC	11.0	87-ONT	6.3
54-ALAS	8.6	44-NY	5.5
65-SASK	8.0	60-QUE	4.9
64-MAN	7.6	30-MONT	3.7
53-ALAS	4.7	59-NB	3.6

[1]Any two means not included within the same line appearing to the right of each ranking of sources are significantly different.

western sources (Table 13). Von Rudloff (1967) also found lowest concentrations of β-pinene and highest concentrations of limonene in leaf-oil samples from western Canada.

The cluster analysis described by Flake and Turner (1968) and applied to the terpene date further clarifies the east-west pattern of differentiation of monoterpenes in white spruce. In any taxonomic approach an attempt is made to cluster or group the populations sharing the largest number of characters, or those which appear to share a common gene pool. This numerical classification procedure identifies these clusters and orders them into a hierarchy. The hierarchy of aggregations constructed by this method is illustrated in the form of a contour map for the 16 white spruce sources in Figure 8. In constructing this hierarchy the monoterpenes of relatively high variability were given less emphasis (Flake, von Rudloff, and Turner, 1969).

The sources of white spruce most similar in terpene composition were 1664 and 1665 from Manitoba and Saskatchewan (level 1). The next most similar sources were 1654, 1664, and 1665 from Alaska, Manitoba, and Saskatchewan, respectively (level 2), followed by sources 1657 and 1669 from Labrador and Minnesota (level 3). Aggregations at successively lower levels tend to group additional sources into distinct western and eastern

Table 13.--Ranking of white spruce sources by percentages of 3-carene and beta-phellandrene.[1]

3-carene		Beta-phellandrene	
Source	Percent of Oleoresin	Source	Percent of Oleoresin
28-SDAK	2.7	59-NB	4.1
87-ONT	2.5	77-BC	2.8
30-MONT	2.2	57-LAB	2.7
59-NB	1.8	69-MINN	2.3
44-NY	1.7	30-MONT	2.3
76-MICH	1.5	86-ONT	2.0
57-LAB	1.5	44-NY	1.9
77-BC	1.2	60-QUE	1.8
60-QUE	1.2	87-ONT	1.6
31-MAN	1.1	76-MICH	1.6
86-ONT	0.9	31-MAN	1.0
64-MAN	0.9	53-ALAS	0.6
54-ALAS	0.8	64-MAN	0.2
65-SASK	0.6	65-SASK	0.2
69-MINN	0.6	53-ALAS	0.1
54-ALAS	0.3	28-SDAK	0.1

[1]Any two means not included within the same line appearing to the right of each ranking of sources are significantly different.

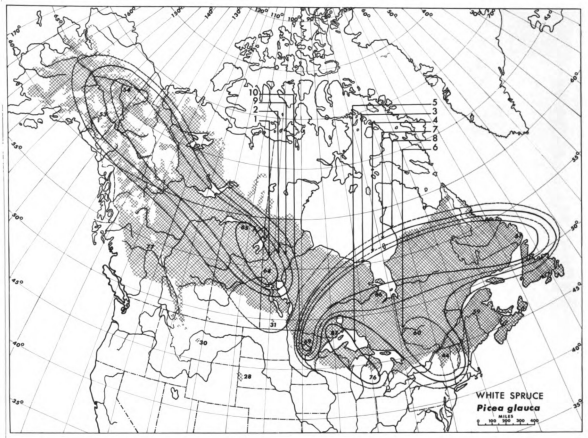


Figure 8.--White spruce aggregation contours utilizing weighted characters. Geographic origins are represented by source number, omitting the first two digits 16.

populations divided roughly by a north-south line between southern Manitoba and Minnesota. But a few sources did not follow this pattern. The New Brunswick source 1659 had extremely high or low concentration for three of the four variable terpenes. South Dakota source 1628 may be different because of its isolation; it is often considered to be a population separated from the main body of spruce subsequent to the Pleistocene glaciation. The British Columbia source 1677 and Montana source 1630 may possibly be influenced by gene exchange with Picea engelmannii which occurs in both areas (Garman, 1957 and Taylor, 1959).

Habeck and Weaver (1969) reported that limonene and β-phellandrene are important terpenes in contrasting white and Engelmann spruce and that the amounts of limonene are lower and the amounts of β-phellandrene are higher in Engelmann spruce than in white spruce. Ogilvie and von Rudloff (1968) also report a reduction in limonene and an increase in 3-carene in the leaf oils of spruce at high elevations in Alberta where Engelmann spruce dominates compared to low elevations where white spruce is the dominant species.

The Montana and British Columbia sources of white spruce are lower in limonene, higher in β-phellandrene, and generally higher in 3-carene than the other western sources of white spruce (Table 10). This lends support to the above mentioned hypothesis of gene exchange with Picea engelmannii.

The validity of the aggregations obtained by the cluster analysis is demonstrated in the results when compared to those obtained by utilizing an analysis of variance and Duncan's new multiple range test applied to source means of individual terpenes (Tables 12 and 13). However, the cluster analysis more clearly defines the divergence of the sources from Montana, South Dakota and New Brunswick and also reveals a great deal of similarity in the terpene composition of adjacent sources such as sources 1664 and 1665 from Manitoba and Saskatchewan, 1676 and 1687 from Michigan and southwest Ontario and 1660 and 1644 from Quebec and New York.

Both methods of analysis indicate that white spruce from the eastern portion of its range differs in terpene composition from western sources. Nienstaedt (1968) reported on growth characteristics of the same series of white spruce seed sources tested at 14 locations in North Dakota, Minnesota, Wisconsin, Michigan, Maine, and New Brunswick. He found that seed sources from Labrador, Alaska, Saskatchewan, and other northern areas grew more slowly than those from the Lake States, Quebec, and Ontario. British Columbia and Montana sources were also slow growing. Nienstaedt suggested that the species may be distributed as two clines extending from the Lake States-Ontario area northwestward into Canada west of Hudson Bay and northeastward into Labrador. My data appears to

substantiate this hypothesis for a general variability pattern in white spruce considering both adaptive traits such as growth and survival and relatively non-adaptive traits such as the monoterpenes.

It is not possible to explain the genetic differentiation in monoterpenes between eastern and western white spruce. No specific adaptive function has been associated with the monoterpenes, but it may be that the adaptive significance of monoterpenes is hidden and will become clear with further study. It is also possible that the difference in terpene composition was caused by selection pressure acting upon genes for growth rate or other characters related in some manner to genes for monoterpenes. However, there is no present evidence linking monoterpene composition to growth traits.

The Pleistocene glaciation obliterated white spruce over much of its current range and uninterrupted evolution has not proceeded for long periods of time. It is possible that the apparent lack of gene exchange and the differentiation of monoterpenes between eastern and western white spruce may reflect the pattern of repopulation following glacial retreat (Figure 8). Halliday and Brown (1943) suggest that white spruce was able to survive the maximal or Illinoian glaciation and the later Wisconsin glaciation in many refugia south of the ice and in the unglaciated Yukon Valley. Perhaps a third

refuge occurred along the exposed Atlantic shelf and parts of Prince Edward Island, Labrador and Newfoundland (Wright, 1955). The large western population of white spruce may have been derived from common ancestors isolated in the Yukon Valley although migration from a refuge further south on the eastern slopes of the Rocky Mountains may be more likely (Löve, 1959). Similarly the pattern of monoterpene composition in the eastern part of the range could be explained by derivation from ancestors existing both in the exposed Atlantic coastal area and south of the maximum ice extent in the Northeast and North Central United States. Tobolski (1968) also attributed much of the variation patterns in monoterpenes of Scotch pine (Pinus sylvestris) to remigration patterns related to the Pleistocene glaciation.

Mode of Inheritance

The use of any character for taxonomic purposes is based on the assumption that the character is under genetic control. However, determination of the mode of inheritance of monoterpene compounds may also be important if they are to be used for taxonomic purposes. In order to obtain this type of information for white spruce, frequency distributions were compiled for each major monoterpene (Figure 9). Values were plotted for 150 individual trees used in this study.

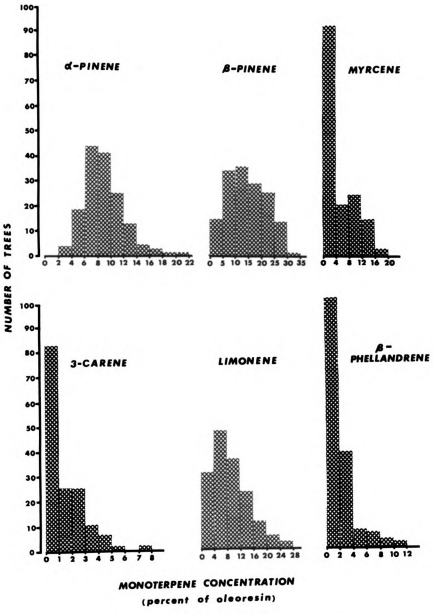


Figure 9.--Frequency distributions of the six major cortical monoterpenes in white spruce.

The distributions for α- and β-pinene from the cortical oleoresin are normal suggesting multiple gene control. It is also possible that levels of these terpenes are regulated by a few genes with the normal pattern of distribution created by environmental modifications. Hilton (1968) reached the same conclusions of multigenic control of these two terpenes in eastern white pine (Pinus strobus). However, Tobolski (1968) reported a trimodal distribution pattern for β-pinene in the cortex of Scotch pine (Pinus sylvestris). Control of β-pinene in cortex gum of slash pine (Pinus elliottii) was postulated to be contolled by relatively few genes (Squillace and Fisher, 1966).

The distributions of limonene, 3-carene, myrcene, and β-phellandrene are definitely skewed rather than normal. Although distinct classes are not evident, the latter three terpenes exhibit a largely bi- or trimodal pattern. Squillace and Fisher (1966) reported a similar distribution for myrcene and β-phellandrene in the cortical tissue of slash pine. Tobolski (1968) and Hilton (1968) reported bi- or trimodal distributions for 3-carene, myrcene, limonene, and β-phellandrene in Scotch pine and eastern white pine, respectively. These workers postulated control of these terpenes by a few genes. However, several interpretations of frequency distributions are possible. Control by few genes or by

several genes with a low plus gene frequency at the several loci could account for the skewed distributions. Only the control of 3-carene by a single gene pair in the cortex of western white pine (Pinus monticola) has been determined from the data of a 2-parent progeny test. Parent-progeny relationships obtained from selected crosses are necessary to confirm the exact nature of the inheritance of monoterpenes in spruce.

Relationship Between Monterpenes

Elucidation of biochemical pathways involved in monoterpene synthesis depends on isolation of the specific enzymes involved and experiments in vitro. However, statistical correlations between monoterpenes may yield information to be utilized in future research. Simple correlations were calculated between the six major monoterpenes in white spruce and between 3-carene and terpinolene (Table 14). If such correlations are confirmed in other coniferous species interpretation of their biological meaning may be possible.

Positive and negative correlations could occur merely as a result of sampling genetically distinct geographic sources. This is well illustrated in white spruce. The significant correlations between β-pinene and limonene and β-phellandrene, 3-carene and limonene, and limonene and β-phellandrene reflect the genetic

Table 14.--Simple correlations between monoterpenes for 150 trees from 16 geographic sources of white spruce.

	Monoterpenes					
	α-Pinene	β-Pinene	Myrcene	3-Carene	Limonene	β-Phellandrene
	----------Correlation Coefficient (r)---------					
β-Pinene	-.02					
Myrcene	-.45**	-.52**				
3-Carene	-.14	.11	-.20			
Limonene	-.05	-.59**	.35**	-.24*		
β-Phellandrene	-.05	.31**	-.18	.17	-.57**	
Terpinolene	--	--	--	.48**	--	

*,** Significant at the 5 and 1 percent levels, respectively.

-- Not calculated

differentiation in monoterpene composition between eastern and western white spruce. Among the other significant correlations only the positive correlation between 3-carene and terpinolene is consistent with data from other species. Tobolski (1968) obtained a significant correlation between these two compounds in Scotch pine. Hilton (1968) also found this relationship in cortical oleoresin of eastern white pine. This relationship may be due to a common precursor or one terpene may be the precursor for the other.

The significant negative correlation between α-pinene and myrcene was also obtained in eastern white

pine (Hilton, 1968). However, Tobolski (1968) found a positive correlation between these two terpenes in Scotch pine. In addition, the correlation between myrcene and α-pinene was also positive in wood oleoresin of Douglas fir (Hanover and Furniss, 1966). It appears that most of these correlations are quite inconsistent between species of Pinus and between some of the pines and white spruce.

CHAPTER III

GENETIC VARIATION IN THE MONOTERPENE COMPOSITION OF RED SPRUCE (*PICEA RUBENS*)

A study of the variability and genetic differentiation of the monoterpenes in red spruce (*Picea rubens*) offers the opportunity to associate the differentiation of these compounds with morphological dissimilarity due to natural hybridization. Geographic seed sources of red spruce planted in a common environment have exhibited considerable variation in growth characters and morphological traits, apparently due to hybridization with black spruce (*Picea mariana*) in areas where these two species are sympatric (Morgenstern and Farrar, 1964; Morgenstern, 1968; and Roche, 1969). Most populations of red spruce sympatric with black spruce occurring from Maryland to Pennsylvania north to the limits of red spruce have been affected by introgressive hybridization which has produced genotypes having a selective advantage on new habitats produced by glaciation.

Previous analysis of the terpene composition of red spruce has been limited to the study of leaf oils in two geographic sources of red spruce (von Rudloff, 1966)

and a genus-wide survey of the essential oil composition in a limited number of red spruce grown in an arboretum in Europe (von Schantz and Juvonen, 1966). Neither of these studies were designed to assess the range of variability in terpene composition associated with geographic source. The work described here is a systematic population analysis of a medium-wide ranging species, red spruce, using the monoterpenes which occur in the cortical oleoresin.

Materials and Methods

Cortical oleoresin samples were collected from 14 geographic origins of red spruce planted in a replicated growth test at Valcartier, Quebec, Canada (Figure 3). This plantation is maintained by the Forest Research Laboratory, Department of Forestry and Rural Development, Ste. Foy, Quebec. The plantation was established in 1959 with 2-2 stock and was 14 years old at the time of sampling. The smallest and largest healthy trees of each source were sampled from each of five replicates for a total of ten trees of each source.

Samples of oleoresin were taken over a 2-day period in late June, 1969. Small incisions were made into the phloem of the 1-year-old internode of the main stem. Ten µl samples of the exuded oleoresin were immediately drawn into calibrated 20 µl capillary tubes.

The capillary tubes containing the samples were placed in sealed centrifuge tubes and refrigerated until analysis.

All samples were analyzed quantitatively by gas-liquid chromatography soon after collection. The details of the analysis are described in Chapter II. Monoterpene composition is expressed as a percentage of the oleoresin, and the total terpene content was obtained by summing all values for the individual terpene concentrations except those of camphene and γ-terpinene which occurred only in very low concentrations. An analysis of variance was performed on each set of data. Degrees of freedom were 13, 4, and 52 for origin, replicate, and error, respectively.

Results and Discussion

Nine monoterpenes were found in the cortical oleoresin of red spruce, 3-carene being consistently present in large amounts. α - and β-pinene, terpinolene, and myrcene were the other terpenes present in high concentration (Table 15). Camphene, limonene, β-phellandrene, and γ-terpinene each accounted for less than 2 percent of the oleoresin.

Von Schantz and Juvonen (1966) also found a high 3-carene content to be characteristic of red spruce branch oils. α- and β-pinene were the only other monoterpenes present in amounts greater than 5 percent. However, these authors report a low content of 3-carene

Table 15.--Cortical monoterpene concentration and growth characteristics for 14 geographic origins of red spruce.

Geographic Origin	Monoterpene						Growth Characteristics (Roche, 1969)		
	α-Pinene	β-Pinene	Mrycene	3-Carene	Terpinolene	Total Monoterpenes	Height[1] at Age 14	Date of Flushing	Trees Damaged by Winter Drying
	Percent of Oleoresin						Meters	Days After June 1	Percent
2019-NC	3.6	7.0	1.3	25.4	2.2	41.3	1.09	21.5	96
2020-WV	4.0	6.5	1.3	25.4	2.1	41.0	.71	26.5	78
2021-PE	4.8	6.8	1.5	22.0	1.8	38.9	1.27	21.0	86
2022-MS	4.6	6.5	1.0	24.9	2.0	40.6	.94	24.0	80
2024-NY	2.8	5.8	1.3	26.1	2.1	39.8	.98	24.0	80
2030-MA	3.9	6.8	1.2	26.9	2.2	42.9	.94	25.5	78
2031-NH	3.4	6.8	2.0	22.0	2.3	38.5	1.03	20.0	82
2032-QE	4.1	7.2	1.3	23.9	2.1	41.6	1.17	16.5	86
2033-QE	3.4	6.3	1.3	22.7	1.9	37.6	1.45	12.5	36
2100-NS	3.9	6.0	1.0	22.5	1.8	36.9	.98	22.5	90
2101-NS	4.0	6.6	1.2	26.0	2.0	41.6	1.17	18.0	82
2102-NB	4.7	7.7	1.3	26.0	2.1	43.7	.89	25.0	78
2103-NB	4.3	7.3	1.1	25.6	1.9	42.1	1.20	17.0	70
2505-NB	3.7	5.0	.9	23.5	2.0	36.9	1.46	16.0	56

[1]The F-value for height was 8.54 which was significant at the .001 level of probability.

in the needle oils of red spruces. Von Rudloff (1966) also found that the needle oils of red spruce contain a small percentage of 3-carene. In a study designed to compare monoterpene concentrations in the xylem, cortex, and foliage of Scotch pine (Pinus sylvestris) Tobolski (1968) found means of 3-carene concentration to be 8.6 and 32.6 percent of the total monoterpenes in needles and cortex respectively.

Apparently 3-carene occurs in higher concentration in cortex oleoresin than in needle oils. Differences in the levels of the other monoterpenes in the cortex, xylem, and needles of Scotch pine indicate that enzyme levels, enzyme activities, or the physiological conditions under which monoterpenes are synthesized and interconverted may vary from one tissue to another (Tobolski, 1968).

There were no significant differences in monoterpene concentration between geographic sources of red spruce. The F-values for each monoterpene are as follows:

Monoterpene	F-value
α-Pinene	1.12
Camphene	1.43
β-Pinene	.27
Myrcene	.88
3-Carene	.96
Limonene	.15
β-Phellandrene	1.38
γ-Terpinene	1.16
Terpinolene	1.35

Moreover, the individual tree variation in monoterpene concentration was much less than that encountered in white spruce (Picea glauca). The ranges of concentration for the major monoterpenes of red spruce are presented in Table 16. The lack of variability in monoterpene concentration is in contrast to the great deal of variability exhibited by these same sources of red spruce in growth characteristics (Table 15).

Red spruce and black spruce are considered to be closely related species (Wright, 1955). Examination of the leaf oils of red and black spruce by von Rudloff (1966) indicated that only minor components occurring in amounts too small for positive identification vary between these two species. Von Schantz and Juvonen (1966) also point out the extreme similarity in terpene composition in the needle oils of red and black spruce. The lack of variability in the monoterpene concentration of the cortical oleoresin between geographic sources of red spruce despite the hybrid origin of some of the sources supports the theory of a close phylogenetic relationship.

To test this relationship more directly a total of 30 black spruce from one plantation in southern Michigan and two widely separated natural stands in Upper Michigan were sampled for monoterpene content in the same manner described for red spruce.

Table 16.--Ranges of cortical monoterpene concentration in 14 geographic origins of red spruce.

Geographic Origin	Monoterpene				
	α-Pinene	β-Pinene	Myrcene	3-Carene	Terpinolene
	----------Percent of Oleoresin----------				
2019-NC	2-7	5-11	1-2	19-30	2-3
2020-WV	3-6	4-11	1-2	21-32	2-3
2021-PE	3-10	2-15	1-3	11-36	1-3
2022-MS	2-13	4-12	0-1	17-32	1-3
2024-NY	1-5	4-9	1-2	17-32	1-3
2030-MA	2-8	3-11	1-2	19-32	2-3
2031-NH	2-5	4-9	1-2	9-30	2-3
2032-QE	2-7	6-9	1-2	17-27	1-3
2033-QE	1-7	2-13	1-2	17-27	1-2
2100-NS	2-10	3-10	1-2	15-29	1-2
2101-NS	1-7	3-14	0-1	21-41	2-3
2102-NB	2-8	4-12	1-2	22-39	2-3
2103-NB	2-8	2-19	0-2	21-30	2-3
2505-NB	1-7	2-12	0-1	19-31	1-2

The same monoterpenes were found in the cortical oleoresin of both species. The means and ranges in concentration of the principle monoterpenes of black spruce are presented in Table 17. The monoterpene concentrations of black and red spruce are compared in Table 18. This table is presented as percent of monoterpene because black spruce had a much lower total monoterpene content than red spruce.

The concentrations of six monoterpenes differed between red and black spruce. Sources 2033-Quebec and 2505-New Brunswick resembled black spruce the most in phenolic content and growth characters. However, they showed no greater resemblance to Michigan black spruce in terpenes than did other sources of red spruce.

Relationship of the Monoterpene Composition of Red, Black and White Spruce

Red and black spruce exhibit a much greater degree of similarity in terpene composition to each other than to white spruce. The means and ranges of cortical monoterpenes in red, white, and black spruce are summarized in Table 19. The amounts of α- and β-pinene, myrcene, limonene, and β-phellandrene were generally greater in white spruce than in black or red spruce. Conversely, the amounts of 3-carene and terpinolene were greater in the latter two species. In addition α-terpinene was

Table 17.--Means and ranges of cortical monoterpene concentration in 1 plantation and 2 natural stands of black spruce in Michigan.

Source	Monoterpene				
	α-Pinene	β-Pinene	Myrcene	3-Carene	Terpinolene
	----------Percent of Oleoresin----------				
Kellogg Forest					
mean	5.2	3.1	1.6	14.5	1.2
range	2-10	2-5	1-2	10-24	1-2
Skanee, Mich.					
mean	3.4	2.5	1.0	14.0	.6
range	1-11	1-4	1-2	10-20	0-1
Watersmeet, Mich.					
mean	2.3	2.4	1.0	12.1	.5
range	1-5	1-4	1-2	7-18	0-1

Table 18.--Comparison of cortical monoterpene concentrations in red and black spruce.

Monoterpene	Species	
	Red Spruce	Black Spruce
	-----Percent of Monoterpene------	
α-Pinene*	11.26	17.98
Camphene*	1.13	.83
β-Pinene*	18.72	13.46
Myrcene*	2.48	4.06
3-Carene	54.51	54.85
Limonene*	1.50	1.02
β-Phellandrene	2.33	2.06
γ-Terpinene	1.31	1.04
Terpinolene*	6.79	4.29

*Significantly different at the 5 percent level.

Table 19.--Means and ranges of cortical monoterpene concentration in white, red, and black spruce.

Monoterpene	Species					
	White Spruce		Red Spruce		Black Spruce	
	Mean	Range	Mean	Range	Mean	Range
	----------Percent of Oleoresin----------					
α-Pinene	8.9	2-21	4.0	1-13	3.6	1-11
β-Pinene	14.4	1-31	6.6	2-19	2.7	1-5
Myrcene	5.7	1-29	1.2	0-3	1.2	1-2
3-Carene	1.3	0-8	24.6	9-41	13.5	7-24
Limonene	9.0	0-27	.3	0-2	.2	0-1
β-Phellandrene	1.6	0-11	.8	0-2	.4	0-1
Terpinolene	.1	0-1	2.0	1-3	.8	0-2

found only in white spruce although its presence in trace amounts may be obscurred by the large amounts of 3-carene in red and black spruce. On the other hand, γ-terpinene was only found in red and black spruce.

It appears that the monoterpene composition of red and black spruce may have been established as a result of common ancestry and has undergone relatively little differentiation despite differences in the climatic adaptation of the two species, black spruce being a strictly boreal species while red spruce is adapted to cool-temperate climates. On the other hand, differences in monoterpene composition between red spruce and white spruce, and black spruce and white spruce is indicative of the closer phylogenetic relationship of white spruce to western species of spruce (Wright, 1955).

Inheritance of Monoterpenes

A few workers have attempted to obtain information on the mode of inheritance of monoterpenes by parent-progeny relationships and construction of frequency distributions. Determining the mode of inheritance of the monoterpenes is almost imperative if the monoterpenes are to be used as taxonomic criteria for establishing phylogenetic relationships.

I have constructed frequency distributions for the major terpenes except terpinolene in red spruce.

These frequency distributions are presented in Figure 10. Values were plotted for 140 individual trees used in this study. For comparative purposes the same scale was used in constructing frequency distributions for both red and white spruce except for the distribution of 3-carene (Figures 9 and 10).

The normal curves encountered for α- and β-pinene in white spruce are not readily apparent in red spruce. However, expansion of the scales for these two terpenes results in normal curves with the greatest frequency occurring at a much lower value than in white spruce. The larger concentrations of α- and β-pinene in white spruce than in red spruce has already been pointed out. Differences in the distributions of α- and β-pinene in white and red spruce may be genetic, or modifiers of the genetic expression may be different in each species.

Modification of the genetic system was encountered in the terpene analysis of hybrids between lodgepole pine (Pinus contorta) and jack pine (Pinus banksiana) (Zavarin, et al., 1969). The pinenes are the most prevalent terpenes in jack pine while β-phellandrene is quantitatively the most important terpene of lodgepole pine. Failure to recover the original amounts of β-phellandrene in the F_2 and F_3 generations despite the simple inheritance proposed indicated that the introduction of competing enzymatic systems producing large amounts of pinenes could lower

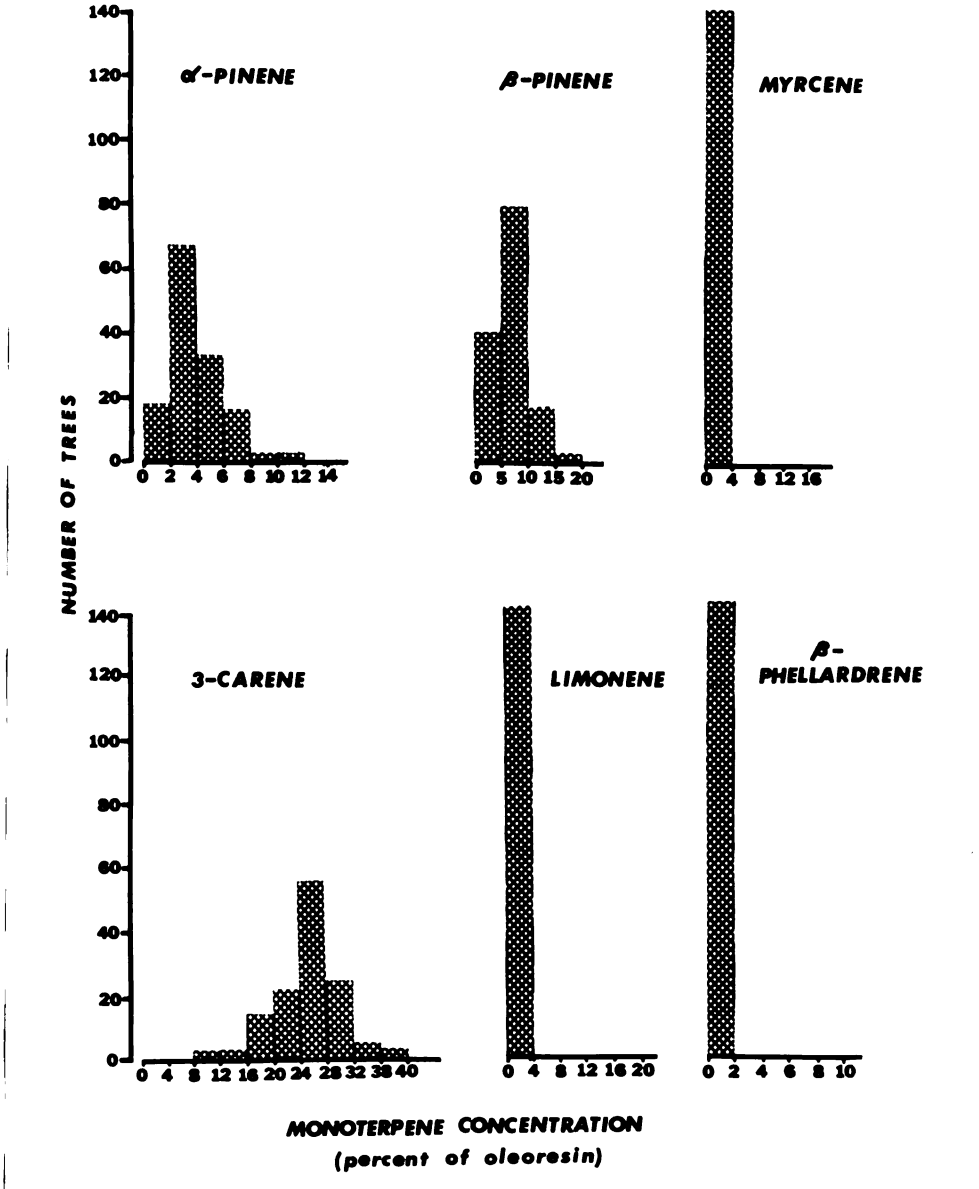


Figure 10.--Frequency distributions of the major cortical monoterpenes in red spruce.

the β-phellandrene level. If this hypothesis is true the genetic background can be very important in the expression of genetically identical systems.

On the other hand, the distributions of myrcene, limonene, and β-phellandrene appear to be represented by a single class or possibly a single genotype in red spruce. If genotypes with the ability to synthesize large amounts of these three terpenes exist or have existed in red spruce, then intense selection against these genotypes must have occurred. This would be the only way to explain their extremely low frequency. On the other hand, the ability to synthesize these terpenes in large amounts may never have occurred in the species. This could be due to an absence of the necessary genes or the occurrence of some species-wide modifier system.

The distribution of 3-carene in red spruce is the most puzzling. The distribution of 3-carene in white spruce is definitely abnormal. A number of authors have reported 3-carene to be controlled by a small number of genes in pine (Hanover, 1966c, Hilton, 1968, and Tobolski, 1968). However, in red spruce 3-carene is present in a wide distribution approximating a normal curve at a high level. Moreover, there is no overlap in the concentration of 3-carene found in red spruce and white spruce. No explanation for this difference is readily apparent.

More work is needed to clarify the inheritance patterns of the monoterpenes in spruce before their full use as taxonomic criteria can be evaluated. In addition, the modifying systems which appear to be important in the genetic expression of the terpene concentration in spruce and pine must be clarified. Perhaps some inroads can be made by studies of the oleoresin components other than monoterpenes such as the resin acids.

Relationships Between Monoterpenes

Calculation of simple correlations between monoterpenes in white spruce yielded little biological information due to lack of confirmation in other species. I did not calculate simple correlations for the monoterpenes of red spruce. The reasons for this were two-fold. First the lack of meaningful results indicates many factors may be involved in the expression of terpene concentration including genetic differentiation encountered in sampling widely separated geographic sources. Secondly and most important is the lack of variability in the monoterpenes of red spruce. If the number of genotypes has been severly restricted, correlations between monoterpenes may reflect only the error involved in measurement whether it be experimental, environmental or physiological.

One significant fact that should be mentioned regarding correlations between terpenes is the confirmation

of the high positive correlation between 3-carene and terpinolene. Red and black spruce have higher concentrations of 3-carene than white spruce. As can be seen in Table 18 this difference in 3-carene concentration is accompanied by a similar difference in the concentration of terpinolene.

CONCLUSIONS AND RECOMMENDATIONS

The large numbers of phenolic compounds in spruce foliage and the occurrence of species-specific compounds in white, red, and black spruce warrant their use as taxonomic criteria. Their greatest value lies in the detection of introgressive hybridization. Hybridization in specific sources or populations of red and black spruce can be detected by paper chromatography of phenolic compounds and calculation of chemical affinity values between each species involved.

However, the greatest practical value lies in the species specificity of compounds 4 and 40. Due to their high concentration in every black spruce sampled, a single red spruce could be sampled and detected as the pure species, or an introgressant. This is not possible with calculations based on total phenols. A compound occurring in 1 sample of 100 collected from a pure species is considered to be species-specific as long as it is not present in the other species. Therefore, large samples would have to be collected to make sure that genotypes in low frequency would be represented.

Unfortunately, compounds 4 and 40 are the only ones which can be estimated quantitatively by visual

estimates alone. Therefore hybridization and introgression in black spruce cannot be detected. In addition F_1 hybrids, which may be the most valuable for future breeding and improvement work, cannot be detected either. However, the use of compounds 4 and 40 may have practical value for detecting hybrids which have introgressed into red spruce. Red spruce is a species which is very difficult to plant in exposed habitats. However, the introgressant hybrids have silvicultural potential in that they grow faster, exhibit little winter injury in northern climates, and can be planted in open sites. In addition, Manley and Fowler (1969) have found that in the red-black spruce complex, the closer an individual tree approximated red spruce the greater the severity of spruce budworm defoliation.

Therefore, compounds 4 and 40 can serve as valuable markers for selecting breeding stock both for increased growth and survival and for budworm resistance. Paper chromatographic techniques are fairly rapid and both compounds can be detected by simple examination in ultraviolet radiation. However, the value of these compounds would be greatly magnified with the development of techniques to analyze crude phenolic extracts by gas-liquid chromatography. A procedure for utilizing gas chromatography for analysis of phenolic glycosides in gymnosperms has been described by Rast et al. (1964). In addition to

greater speed of analysis, gas chromatography provides a means for rapid and accurate quantitative analysis.

In contrast to the phenolic compounds, species differences in monoterpenes are largely quantitative, and separation of species relies heavily on differences in gene frequency. Many of the monoterpenes may be simply inherited and large sample sizes are needed to detect these differences.

Monoterpenes may be more suitable for detecting the phylogenetic relationships between species. Red and black spruce are more similar to each other than to white spruce in monoterpene concentration. In phenolic compounds black spruce is more similar to white spruce. This is true even if black spruce is compared to introgressed populations of red spruce. The monoterpenes may have a lower adaptive value than phenolic compounds and therefore would remain unchanged over longer periods of time, especially in the absence of genetic drift as an evolutionary process.

On the basis of monoterpene composition, hybrids of red spruce with black spruce could not be differentiated from pure red spruce. This may be due to a similarity of terpene composition between black spruce from the eastern portions of its range and red spruce. When range wide provenance tests of black spruce become available, sources of black spruce from the east and the west

can be examined for their monoterpene content and the hybrid situation in red spruce may be clarified.

Although the monoterpenes were unsuitable for detecting hybridization between red and black spruce, they may still be of value in hybridization studies in other spruce species. The hybridization between white and Sitka spruce would be a good complex for future study. This study may provide additional information concerning the phylogenetic relationships between spruce species of North America.

The relative values of phenolics and monoterpenes for assessing intraspecific variation cannot be compared. The portion of this study involving phenolic compounds was restricted to the Northeast. Geographic variation in the phenolics of red and black spruce was obscured by interspecific hybridization. Differences within white spruce were largely random with distant populations often being more similar to each other than those in close proximity. However, there may also be little differentiation in the monoterpenes within eastern white spruce. Only the analysis of more samples from this area can indicate the relative value of each chemical group.

However, it appears that the monoterpenes have a great potential value for assessing intraspecific variation. The general agreement between differentiation in monoterpenes and height growth in white spruce indicates

that these two traits, one of presumably high adaptability, and the other of low adaptability, can complement each other in studies of species evolution.

The causes for the east-west pattern of monoterpene variation in white spruce cannot be defined. I would recommend that terpene studies be conducted in other spruce species which were not so greatly affected by the Pleistocene glaciation. Patterns of variation in these species may suggest some correlation in white spruce. Red spruce does not satisfy this requirement due to small population size and the occurrence of extensive hybridization. The best species would probably be Engelmann spruce as it occupies a fairly large range south of the glaciated areas. However, it is a high altitude species and extrapolation to white spruce may be difficult.

Monoterpenes have a single main advantage over phenolic compounds for use as a taxonomic tool. A great deal more is known about their genetics. However, lack of knowledge concerning the inheritance of phenolics does not preclude their use as taxonomic criteria. Many morphological traits which have been traditionally used in taxonomy are under very obscure genetic control.

However, the large amounts of variation in the phenolic compounds of individual trees suggests that very large samples must be obtained if phenolic compounds are to be used for assessing intra- and interspecific

variation. In addition, the effects of site on variation in phenolic compounds must be determined. Sampling from single plantations probably eliminated much of the variation which is not genetic, but many other studies have been and currently are being conducted in natural stands. Most studies involve sampling only a few trees from each area. My study indicates that any results obtained on this basis may be erroneous.

One of the more important incites gained from this study is the need for meticulous sampling techniques. Tissue age, season of collection, and fresh versus dried foliage resulted in differences in both the number of phenolic compounds present and the presence and absence of compounds. In some cases the number of compounds in common between samples from the same tree were much lower than between species.

The variation in phenolic compounds from foliage of different ages means that internodes must be separated at the time of removal from the tree. Drying of spruce foliage causes all of the needles to be shed and if not previously separated the mixture obtained may give erroneous results. In the same manner collections must be made at the same time of year and samples must be either dried or kept fresh. Comparison of samples from herbarium specimens with fresh material may only lead to false results. Further study is needed to clarify these differences,

which means identifying the compounds involved so that the physiological basis for differences can be determined.

Finally, more work will have to be done concerning the inheritance of monoterpenes. The possible discrepancy between the inheritance of monoterpenes in red and white spruce should be clarified to improve their usefulness as taxonomic criteria. Parent-progeny analyses in both species and the assessment of physiological, genetic, and environmental modifiers need to be done. It does not seem likely that the monoterpenes are under different systems of genetic control in two species of the same genus.

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APPENDIX

Table 20.--Geographic location of red spruce seed sources from which cortical oleoresin samples and foliage samples for analysis of phenolic compounds were obtained.

Source Number	Location	Lat. °N.	Long. °W.
2019	Great Smoky Mts., No. Car.	35°36'	83°27'
2020	Monongohela Nat. For., W. Vir.	38°38'	79°50'
2021	Bear Meadows, Penn.	40°45'	77°45'
2022	October Mts., Mass.	42°22'	73°15'
2024	Essex County, N. York	44°25'	73°40'
2030	Amherst, Maine	44°54'	68°23'
2031	Andorra Forest, N. Hamp.	43°05'	72°07'
2032	Valcartier Forest Exp. Sta., Que.	46°30'	75°20'
2033	Saint Charles de Mandeville, Que.	46°30'	75°20'
2100	Halifax County, N. Scotia	45°12'	62°44'
2101	Digby County, N. Scotia	44°10'	65°54'
2102	St. John County, N. Brun.	45°25'	65°24'
2103	Acadia Forest Exp. Sta., N. Brun.	46°00'	66°20'
2505	Acadia Forest Exp. St., N. Brun.	46°37'	66°40'

Table 21.--Geographic location of black spruce seed sources from which foliage samples for analysis of phenolic compounds were obtained.

Source Number	Location	Lat. °N.	Long. °W.
1792	Dog River, Ontario	49°25'	89°55'
1794	Kapuskasing, Ontario	49°25'	82°25'
1795	Fort William, Ontario	48°20'	89°20'
1796	Newfoundland		
1798	St. Zenon Quebec	46°33'	73°40'
1799	Rimouski County, Quebec	48°	68°
1800	St. Faustin, Quebec	46°08'	74°28'
1801	Nt. Pearl, Newfoundland	47°31'	52°47'
2107	Halifax County, N. Scotia	45°	63°
2108	Hants County, N. Scotia	45°	63°
2109	Sunbury County, N. Brunswick	46°	66°
2110	York County, N. Brunswick	46°	66°
2125	Upper Trout Lake, Wisconsin	45°	89°
2126	Hazelhurst, Wisconsin	45°	89°
2127	Jute Lake, Wisconsin	45°	89°
2377	Maritimes Via Anges		

Table 22.--Geographic location of white spruce seed sources from which foliage samples for analysis of phenolic compounds were obtained.

Source Number	Location	Lat. °N.	Long. °W.
1644	Saranac Lake, New York	44°23'	74°06'
1649	Pittsburg, New Hampshire	44°50'	71°26'
1655	Bradley, Maine	44°50'	68°38'
1657	Port Hope Simpson, Labrador	52°36'	56°26'
1658	Lake Melville, Labrador	53°36'	60°25'
1659	Edmondston, New Brunswick	47°50'	68°21'
1660	Maniwaki, Quebec	46°32'	76°30'
1661	Lake Kenogami, Quebec	48°18'	71°22'
	Quebec Rd. Depot, Quebec	48°13'	71°38'
1662	Ashley Mines, Ontario	48°00'	81°00'
1663	Pembroke, Ontario	45°44'	76°51'
1676	Mio, Michigan	44°	84°
1686	Akimiski Island, Ontario	52°15'	81°40'

Figure 11.--Representative chromatograms of the ether-soluble (left) and butanol-soluble (right) phenolics of white, black, and red spruce.

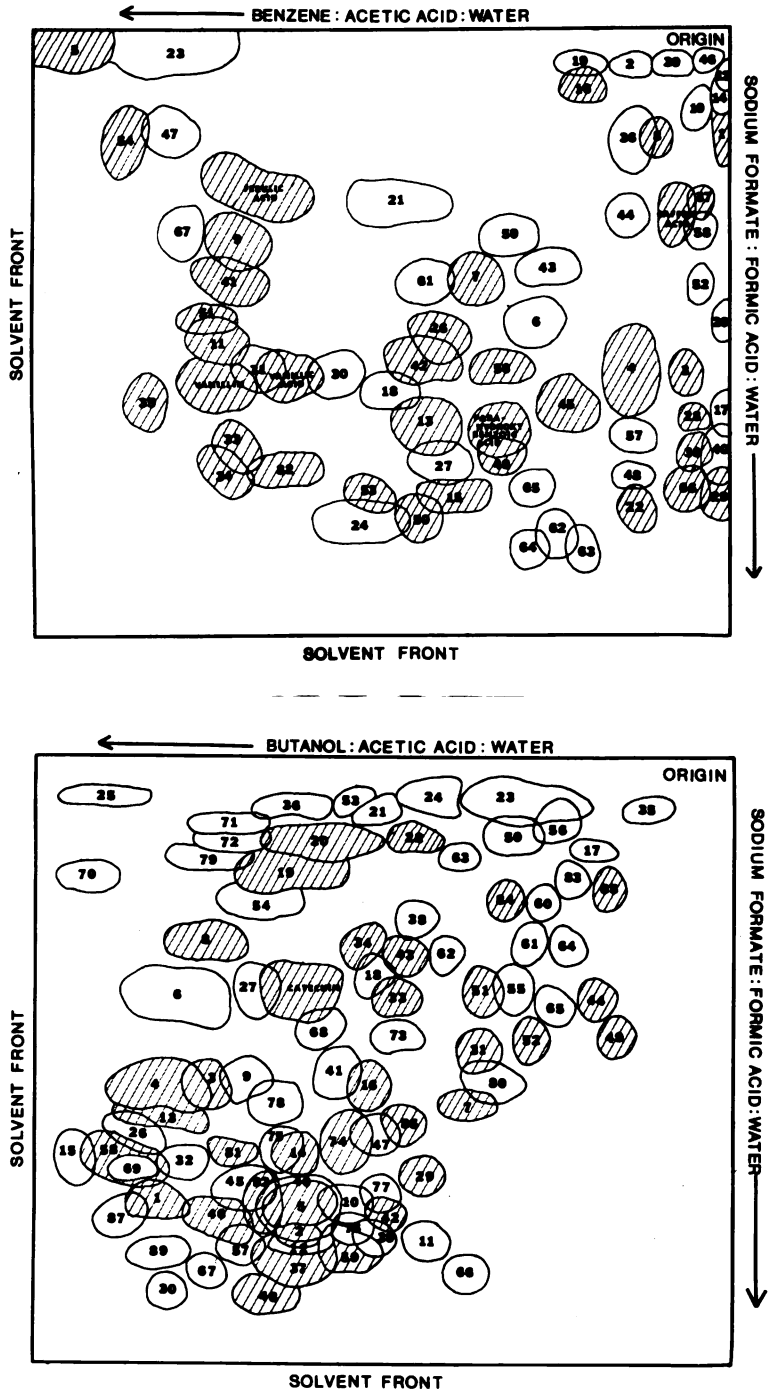

Figure 12.--Composite chromatograms of ether-soluble (upper) and butanol-soluble (lower) phenolic compounds of spruce foliage.

Table 23.--R_f values and color reactions of the ether-soluble phenolic compounds in spruce foliage.[1]

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Benzene, Acetic Acid, Water (6:7:3)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
Ferulic Acid	.66	.24	Bl	-	YB	V
Vanillic Acid	.65	.57	-	-	YB	O
Vanillin	.75	.63	DP	-	YB	OPk
Para-hydroxy-Benzoic Acid	.31	.67	-	-	YB	DY
Caffeic Acid	.07	.25	-	Bl	LB	PGr
1	.00	.09	YG	-	-	YB
2	.09	.00	YO	-	Y	-
3	.05	.54	L	-	YB	M
4	.14	.54	VW	-	LB	BrY
5	.95	.00	O	-	YB	OB
6	.29	.44	BrBl	-	-	-
7	.37	.38	DBl	-	Y	RPk
8	.09	.13	-	-	YB	-
9	.70	.35	G	-	-	OB
10	.04	.09	L	-	-	-
11	.76	.55	DBl	-	-	OPk
12	.00	.04	Bl	-	-	-
13	.46	.68	P	-	YB	O
14	.00	.02	D	-	-	-
15	.41	.81	-	-	YB	Pk
16	.18	.06	-	-	YB	-
17	.00	.72	BrBl	-	-	-
18	.50	.65	DP	-	-	-
19	.20	.00	L	-	-	-
20	.00	.50	LBl	-	-	-

Table 23 (Continued)

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Benzene, Acetic Acid, Water (6:7:3)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
21	.48	.23	Bl	-	-	-
22	.16	.78	-	-	-	YO
23	.81	.00	BlG	-	-	-
24	.51	.85	Bl	-	-	-
25	.00	.13	D	-	-	-
26	.46	.48	DG	-	-	Y
27	.42	.70	D	-	-	-
28	.06	.66	-	-	LB	O
29	.00	.76	-	-	-	YO
30	.57	.56	Y	-	-	-
31	.68	.59	Y	-	-	-
32	.64	.77	-	-	YB	Pk
33	.69	.74	-	-	YB	Pk
34	.72	.80	-	-	YB	O
35	.65	.70	-	-	YB	Pk
36	.11	.14	BlG	-	-	-
37	.04	.22	-	-	Y	-
38	.06	.73	-	-	YB	0
39	.05	.00	BrBl	-	YB	-
40	.00	.72	P	-	-	-
41	.68	.44	-	-	YB	DPk
42	.50	.54	-	-	YB	M
43	.29	.34	Bl	-	-	-
44	.14	.30	LBl	-	-	-
45	.24	.62	-	-	YB	OPk
46	.02	.00	0	-	-	-
47	.81	.15	LG	-	-	-

Table 23.--(Continued)

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Benzene, Acetic Acid, Water (6:7:3)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
48	.08	.70	Bl	-	-	-
49	.32	.75	-	-	-	Pk
50	.50	.82	-	-	-	Y
51	.76	.50	-	-	-	Pk
52	.04	.39	L	-	-	-
53	.58	.81	-	-	-	Pk
54	.90	.20	-	-	-	OPk
55	.03	.60	LB	-	-	-
56	.37	.52	-	-	-	Pk
57	.09	.59	D	-	-	-
58	.02	.29	LY	-	-	-
59	.37	.24	Bl	-	-	-
60	.69	.54	Bl	-	-	-
61	.45	.39	Bl	-	-	-
62	.19	.80	-	-	YB	-
63	.17	.88	Bl	-	-	-
64	.24	.87	Bl	-	-	-
65	.22	.77	L	-	-	-
66	.08	.75	L	-	-	-
67	.78	.35	Bl	-	-	-

[1]Color abbreviations: P = purple; Bl = blue; G = green; W = white; Y = yellow; B = brown; R = red; O = orange; Pk = pink; Bk = black; L = light; D = dark; M = magenta; Br = bright; V = violet; Gr = gray.

Table 24.--R_f values and color reactions of the butanol-soluble phenolic compounds in spruce foliage.[1]

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Butanol, Acetic Acid, Water (4:1:5)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
Catechin	.65	.33	-	L	YB	YB
1	.82	.75	-	-	YB	RO
2	.57	.80	-	-	R	LO
3	.76	.56	-	-	YB	M
4	.79	.55	WV	-	YB	BrY
5	.55	.72	-	-	YB	M
6	.78	.37	Bl	-	-	-
7	.27	.55	-	-	-	Pk
8	.72	.25	Bl	-	-	V
9	.74	.59	-	-	-	-
10	.52	.70	BlG	-	-	-
11	.45	.80	P	-	-	-
12	.56	.84	LY	-	-	-
13	.68	.65	P	-	YB	O
14	.56	.62	G	-	-	Y
15	.88	.72	-	-	-	-
16	.52	.50	DBl	-	-	Pk
17	.23	.07	Y	-	-	-
18	.52	.30	Y	-	-	-
19	.64	.14	DG	-	YB	YB
20	.59	.08	YG	-	YB	-
21	.52	.03	P	-	-	-
22	.49	.06	YO	-	YB	-
23	.27	.00	BrBl	-	PkB	-
24	.40	.00	LBl	-	PkB	-
25	.85	.00	-	-	PkG	PkG

Table 24.--(Continued)

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Butanol, Acetic Acid, Water (4:1:5)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
26	.81	.65	P	-	-	-
27	.68	.36	Bl	-	-	-
28	.40	.92	-	-	-	RO
29	.33	.67	-	-	YB	-
30	.80	.90	-	-	-	-
31	.36	.46	-	-	YB	-
32	.74	.62	Bl	-	-	-
33	.44	.31	-	DB	YB	YB
34	.53	.26	-	-	YB	-
35	.09	.01	Bl	-	-	-
36	.61	.00	LBl	-	-	-
37	.58	.86	-	-	YB	RO
38	.26	.36	Y	-	-	-
39	.37	.76	YO	-	-	-
40	.60	.77	P	-	-	-
41	.58	.47	BlG	-	-	-
42	.41	.73	-	-	-	Pk
43	.40	.27	-	-	YB	-
44	.24	.30	-	-	YB	-
45	.64	.73	Bl	-	-	-
46	.66	.79	-	-	YB	O
47	.45	.60	LBl	-	-	-
48	.57	.90	-	-	YB	R
49	.20	.38	-	-	YB	-
50	.33	.05	P	-	-	-
51	.30	.38	-	-	YB	YB
52	.23	.40	-	-	-	-

Table 24.--(Continued)

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Butanol, Acetic Acid, Water (4:1:5)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
53	.51	.00	PkY	-	-	-
54	.73	.15	LG	-	-	-
55	.29	.32	D	-	YB	-
56	.32	.04	DB	-	-	-
57	.64	.83	-	DBl	-	-
58	.81	.65	-	-	YB	O
59	.50	.83	-	-	YB	O
60	.24	.16	Y	-	-	-
61	.27	.23	D	-	-	-
62	.32	.25	-	-	-	-
63	.34	.10	-	D	-	-
64	.24	.28	L	-	-	-
65	.21	.70	L	-	-	-
66	.22	.84	-	P	-	-
67	.67	.87	Bl	-	-	-
68	.60	.40	LBl	-	-	-
69	.86	.65	O	-	-	-
70	.95	.14	Bl	-	-	-
71	.63	.04	D	-	-	-
72	.75	.04	-	-	Yb	-
73	.46	.33	LBl	-	-	-
74	.56	.61	Y	-	-	YO
75	.59	.60	Bl	-	-	-
76	.54	.76	BlG	-	-	-
77	.48	.70	BlG	-	-	-
78	.67	.54	BlG	-	-	-
79	.75	.11	-	-	-	-

Table 24.--(Continued)

Compound Number	R_f		UV		Diazotized Sulfanilic Acid	2N NaOH
	Butanol, Acetic Acid, Water (4:1:5)	Sodium Formate Formic Acid, Water (10:1:200)	Untreated	NH_3		
80	.35	.53	LBl	-	-	-
81	.73	.65	-	-	-	Pk
82	.62	.71	-	-	-	Pk
83	.20	.13	-	-	-	-
84	.30	.18	-	-	YB	-
85	.37	.54	-	-	YB	-
86	.26	.62	-	P	-	-
87	.85	.82	Bl	-	-	-
88	.21	.15	-	-	-	-
89	.75	.87	Bl	-	-	-

Color abbreviations: P = purple; Bl = blue; G = green; W = white; Y = yellow; B = brown; R = red; O = orange; Pk = pink; Bk = black; L = light; D = dark; M = magenta; Br = bright; V = violet; Gr = gray.

Table 25.--Chemical similarity between geographic sources of red spruce.

	Geographic Source												
	2019 NC	2020 WV	2021 PE	2022 MS	2024 NY	2030 MA	2031 NH	2032 QE	2033 QE	2100 NS	2101 NS	2102 NB	2103 NB
	Percent of Compounds in Common[1]												
2020-WV	92 91	—											
2021-PE	74 73	72 73	—										
2022-MS	78 85	78 84	86 78	—									
2024-NB	85 90	84 88	76 80	85 84	—								
2030-MA	80 96	80 88	72 77	83 88	91 92	—							
2031-NH	86 92	90 86	78 78	90 85	94 90	88 90	—						
2032-QE	76 89	74 87	83 85	90 89	87 83	83 89	84 86	—					
2033-QE	75 78	74 74	90 83	84 78	82 74	78 80	83 80	90 86	—				
2100-NS	81 91	80 92	86 81	92 86	88 88	83 96	88 86	86 89	83 83	—			
2101-NS	76 86	76 84	84 77	82 86	80 85	82 94	80 88	89 89	88 78	87 86	—		
2102-NB	84 89	86 87	80 80	87 89	87 88	89 96	94 89	83 95	85 79	87 92	82 90	—	
2103-NB	80 81	80 75	88 91	85 83	80 80	76 83	81 78	83 84	96 92	80 80	78 84	85 87	—
2505-NB	82 84	76 79	82 88	86 84	81 80	83 86	81 79	93 87	90 80	84 86	90 87	86 87	83 86

[1]The upper values represent affinity values for the ether-soluble fraction, the lower values for the butanol-soluble fraction.

Table 26.--Chemical similarity between geographic sources of black spruce.

	Geographic Source										
	2125-27 WIS	1792 ON	1794 ON	1795 ON	1800 QE	1799 QE	1798 QE	1796 NF	1801 NF	2377 MT	2107-08 NS
	Percent of Compounds in Common[1]										
1792-ON	83 84	—									
1794-ON	90 85	86 84	—								
1795-ON	92 86	79 92	78 86	—							
1800-QE	85 84	94 88	88 84	81 88	—						
1799-QE	88 78	91 78	88 81	87 82	93 79	—					
1798-QE	91 77	88 86	92 84	85 83	93 87	90 84	—				
1796-NF	91 81	81 86	89 83	85 88	87 85	95 80	94 83	—			
1801-NB	83 81	85 80	87 81	82 84	91 86	91 84	88 88	88 82	—		
2377-MT	84 82	87 84	85 81	81 92	86 86	86 81	90 86	87 80	80 84	—	
2107-NS 08	86 78	94 85	90 76	86 82	88 90	91 85	91 88	88 84	89 91	84 83	— —
2109-NB 10	84 86	78 88	85 87	84 82	93 86	89 80	93 88	87 85	84 88	89 80	94 86

[1]The upper values represent affinity values for the ether-soluble fraction, the lower values for the butanol-soluble fraction.

Table 27.--Chemical similarity between geographic sources of white spruce.

	Geographic Source										
	1676 MI	1644 NY	1649 NH	1655 MA	1663 ON	1660 QE	1659 NB	1662 ON	1661 QE	1686 ON	1657 LB
	Percent of Compounds in Common[1]										
1644-NY	90 82	—									
1649-NH	85 86	92 91	—								
1655-MA	85 85	92 91	94 92	—							
1663-ON	87 82	90 90	92 91	96 88	—						
1660-QE	85 90	92 90	90 91	94 90	92 90	—					
1659-NB	89 86	85 90	93 93	94 96	89 87	91 95	—				
1662-ON	86 86	90 87	92 84	95 90	90 89	95 89	92 88	—			
1661-QE	84 79	87 82	95 83	96 85	94 82	92 87	96 89	91 85	—		
1686-ON	92 84	89 87	90 88	88 93	92 85	91 92	91 94	89 86	96 84	—	
1657-LB	86 86	89 84	91 80	94 84	90 89	92 86	88 86	93 90	90 86	88 86	—
1658-LB	85 89	88 92	97 91	88 96	95 90	94 90	94 94	92 92	98 89	94 92	91 88

[1]The upper values represent affinity values for the ether-soluble fraction, the lower values for the butanol-soluble fraction.

Table 28.--Geographic location of white spruce seed sources from which cortical oleoresin samples were obtained.

Source Number	Location	Lat. °N.	Long. °W.
1628	Lead, South Dakota	44°10'	103°55'
1630	Lewiston, Montana	46°48'	109°31'
1631	Douglas Station, Manitoba	49°51'	99°30'
1644	Saranac Lake, New York	44°51'	74°06'
1653	Delta Junction, Alaska	63°45'	144°53'
1654	Fort Yukon, Alaska	66°36'	145°11'
1657	Port Hope Simpson, Labrador	52°36'	56°26'
1659	Edmondston, New Brunswick	47°50'	68°21'
1660	Maniwaki, Quebec	46°32'	76°30'
1664	Channing, Flinflon, Manitoba	54°39'	101°36'
1665	Stony Rapids Settlement, Saskatchewan	59°16'	105°59'
1669	Itaska Co., Minnesota	47°30'	94°00'
1676	Mio, Michigan	44°	84°
1677	Fort McLeod, British Columbia	54°00'	123°00'
1686	Akimiski Island, Ontario	52°15'	81°40'
1687	Port Arthur, Ontario	54°30'	89°30'