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DEVELOPMENTAL VARIATION IN VOLATILE OIL OF BLUE SPRUCE

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DEVELOPMENTAL VARIATION IN VOLATILE OIL OF BLUE SPRUCE

By

Patrick Peder Moore

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ABSTRACT

DEVELOPMENTAL VARIATION IN VOLATILE OIL OF BLUE SPRUCE

By

Patrick Peder Moore

The objectives of this research were to describe the within-tree variation and the developmental control of monoterpenes in tissues and organs of blue spruce. The concentration and composition of the steam volatile oil of the needles, xylem, and bark at three positions in the crown were compared. The seasonal development of the volatile oil in the needles, stems, and cones of blue spruce were also compared. The interrelationships among monoterpenes were studied by correlation analysis of monoterpenes within the same organ and of the same monoterpene between different organs.

Great amounts of variation in monoterpenes were found among different tissues of the same age. The needles were high in santene, camphene, limonene, and oxygenated monoterpenes. The bark was higher in α- and β-pinene and 3 carene. The xylem was primarily composed of α- and β-pinene. Large differences were also found with position in the crown for total monoterpenes for needles and xylem, but not for bark. The concentration of monoterpenes in the xylem increased with height in the crown and decreased with height for needles. The yield of total monoterpenes was highly correlated with the proportion of resin canals to other tissues. Although the proportion of resin canal area was greater at the top of the tree in the xylem than at the

bottom of the tree, the cross-sectional area of an individual resin canal did not increase. The distribution of resin canals within the needle changed with position in the crown, with needles from the base of the tree having a higher proportion of their resin canal volume in the distal 25% of the needle.

There were large differences in the composition of the volatile oil with position in the crown for needles and xylem and smaller differences for the bark volatile oil. There were large among-tree differences for most needle and bark monoterpenes for both the yield of the individual monoterpene per gram of tissue and as a percentage of the total monoterpenes.

The patterns of seasonal development of total monoterpenes were different in needles, stems, and cones. There was no change in the concentration of the total foliar monoterpenes on a dry weight basis during needle elongation, but a rapid increase when elongation ceased, and no changes over the remainder of the growing season. The concentration of total stem monoterpenes increased rapidly immediately after bud break reaching a constant level before stem elongation ceased. There was no constant level reached in the cones on a dry weight basis, but when the concentration of total monoterpenes was calculated as µl/cone there appeared to be a linear increase in monoterpenes over the growing season.

Large amounts of unknown high molecular weight volatile compounds were found in the stem and cone. These compounds showed seasonal variation and significant differences among trees. The major stem and cone unknown compounds had different retention times when chromatographed

on an SE-30 column.

The correlation coefficients between monoterpenes in the same organs were found to change over the growing season for most pairs of compounds. These changes may be significant in the biosynthetic relationship between monoterpenes. The correlations agreed very well with the hypothesized biosynthesis proposed by Zavarin (1970). The concentrations of the total monoterpenes were not significantly correlated between organs. Additionally, with the exception of β-pinene, myrcene, and 3-carene the individual monoterpene levels were not significantly correlated between organs.

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

Products of secondary metabolism present in plants are becoming of increasing interest to researchers in the plant sciences. Many of these compounds have proven useful for chemosystematic and genetic studies as well as being involved in resistance to pests. One such group of compounds, the terpenes, are of special interest for these purposes. Terpenes are compounds that are composed of multiples of C_5 units synthesized in accordance with the "biogenetic isoprene rule" of Ruzicka (1959). They are made from acetyl-Co-A by way of isopentenyl pyrophosphate. Many important compounds have a terpenoid nature, including carotenoids, steroids, and rubber (figure 1). A better understanding of the genetic regulatory aspects of this pathway is needed to facilitate further chemosystematic work.

Monoterpenes are small, fragrant, volatile compounds derived from the terpenoid pathway and composed of two C_5 units. They are a major component of oleoresin produced by many coniferous species. Major monoterpenes found in the needles and cortical oleoresin of blue spruce (Picea pungens Engelm.) are shown in figure 2.

Monoterpenes are commercially important primarily because oleoresin is the base for the production of turpentine (Erickson, 1976; Zinkel, 1975). A new experimental technique may greatly increase production efficiency in the naval stores industry. Treating pines and other species with Paraquat (1,1' dimethyl-4,4'-bipyridinium

Figure 1. Biogenetic pathways of plant terpenes (from Westfall, 1972).

BIOGENETIC PATHWAYS OF TERPENES IN PLANTS

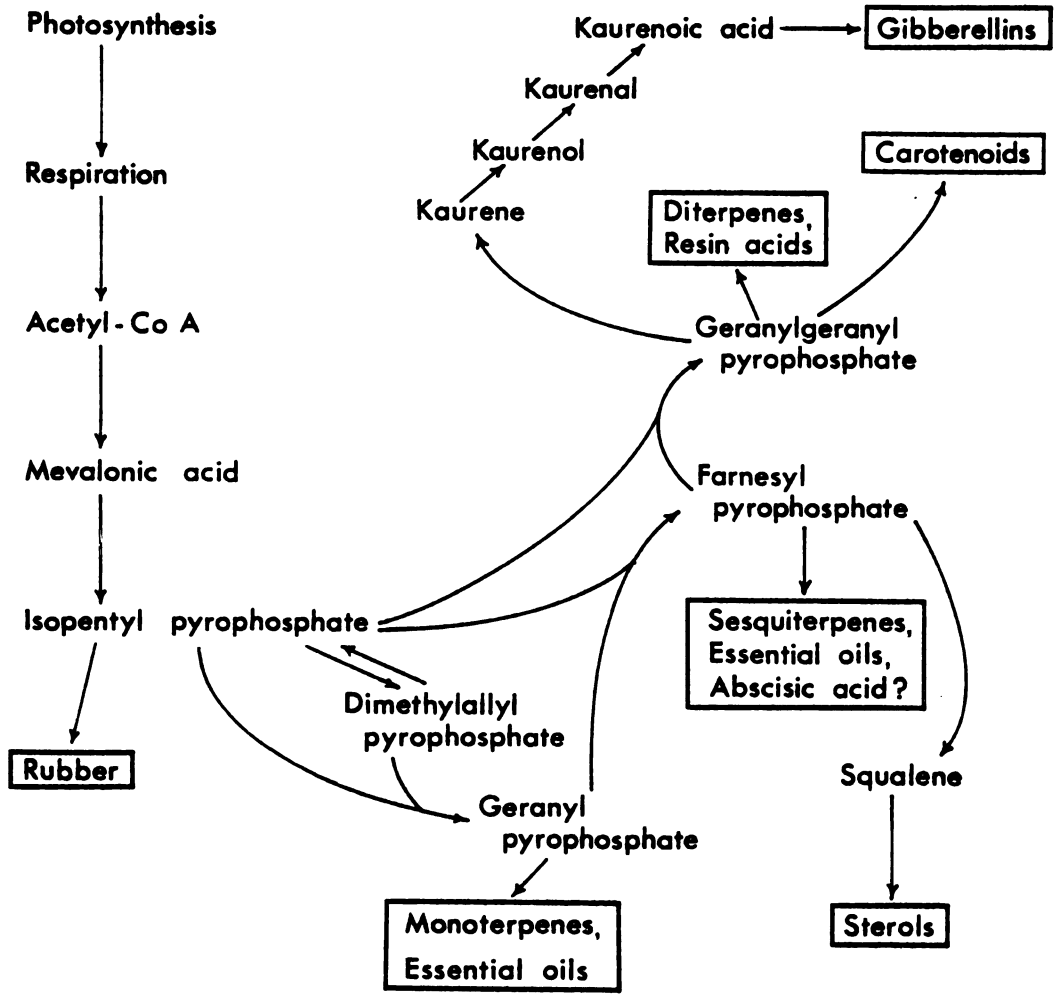


Figure 1.

Figure 2. Chemical structures of major monoterpenes present in blue spruce.

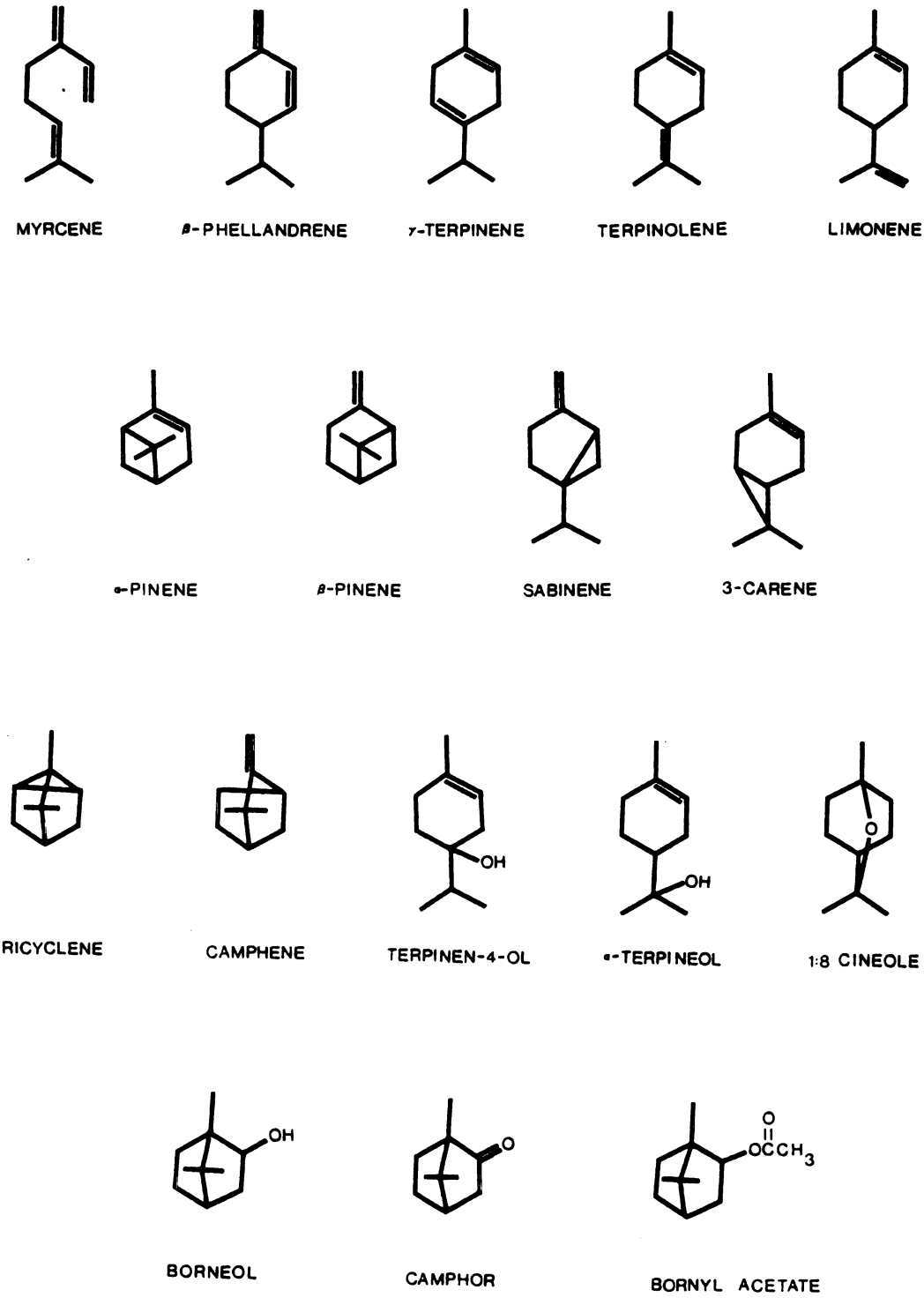

MYRCENE
β-PHELLANDRENE
γ-TERPINENE
TERPINOLENE
LIMONENE
α-PINENE
β-PINENE
SABINENE
3-CARENE
TRICYCLENE
CAMPHENE
OH
TERPINEN-4-OL
OH
α-TERPINEOL
O
1:8 CINEOLE
OH
BORNEOL
O
CAMPHOR
O
OCCH3
BORNYL ACETATE

Figure 2.

dichloride) has been found to cause a substantial increase in the resin content of the wood (Drew, 1976; Drew and Roberts, 1977; Roberts and Peters, 1977). In addition to naval stores there is an important leaf oil industry which was described very early by Schroger (1916).

Monoterpenes occur naturally in the atmosphere (Rasmussen, 1972; Rasmussen and Went, 1965). They are released from plants as vapors and may play an important part in insect and animal behavior toward conifers (Hanover, 1972).

Biological Activity of Monoterpenes

The functional significance of the vast quantities of terpenes found in conifers and other plants is not clear. However, they show biological activity toward a variety of organisms. Monoterpenes have been shown to have an allelopathic effect (Asplund, 1968). The vapors of monoterpenes can be very toxic, with some compounds inhibiting the germination of radish seeds more strongly than HCN. The compounds present in Abies balsamea (L) Mill. have a strong bactericidal effect (Smirnoff, 1972) which could reduce the effectiveness of applied entomopathogenic bacteria.

Monoterpenes inhibit some pathogenic fungi and may be important in tree disease resistance. Rockwood (1973a) reported that resistance to fusiform rust in slash pine (Pinus elliottii Engelm.) was negatively correlated with the level of cortical β-phellandrene. Whitney and Denyer (1969) reported that unsuccessful artificial inoculations of white spruce (Picea glauca (Moench) Voss) roots with six heartrotting fungi had higher resin ratings than roots with successful inoculations. Similarly, Gibbs (1968) found that resistance to Fomes annosus was

related to the resin system of the tree and its ability to produce resin. Cobb et al. (1968) found that vapors of crude xylem oleoresin from ponderosa pine (Pinus ponderosa Laws.) inhibited growth of Fomes annosus and three Ceratosystis species. When individual monoterpenes were tested, Fomes annosus was found to be inhibited by every monoterpene. Monoterpenes were found to reduce the growth of one blue stain fungal species and to stop the growth of another species (Shrimpton and Whitney, 1968). The germination and growth of Diplodia pinea were inhibited by monoterpenes (Chou and Zabkiewicz, 1976). Compounds which were most inhibitory were 3-carene and (+) limonene. The naturally occurring stereoisomer in the host, Pinus radiata D. Don, is (-) limonene, which was much less effective than (+) limonene at inhibiting germination and did not inhibit the growth of the fungus.

The monoterpene composition of a tree may influence animal browsing preference. Radwan (1972) found that steam distilled volatile oils decreased the digestibility of an artificial substrate, measured by in vitro rumen fermentation. Oils from different Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco) clones were found to have different effects on digestibility. Oh et al. (1970) reported that added oils decreased the microbial activity of the deer rumen as measured by in vitro fermentation. Oil from older tissues was found to be more inhibitory than new growth. The older foliage was higher in oxygenated monoterpenes, which were found to be more inhibitory than reduced monoterpenes.

Monoterpenes in host trees appear to affect insect behavior either as attractants or repellants (Hanover, 1975a). They attract flying Douglas-fir bark beetles (Dendroctonus pseudotsugae Hopkins) (Rudinsky,

1966) and may be a precursor of pheromones produced by some Ips beetles (Hughes, 1974). Oleoresin appears to play a role in tree resistance to insect attack. Rudinsky (1966) found that bark beetles could be killed by resin in the insect galleries. Death was probably caused by suffocation. In the laboratory, beetles covered with resin were killed within one minute. Air saturated with resin vapor can also be toxic to insects (Smith, 1961; 1963; Rudinsky, 1966; Coyne and Lott, 1976). Anderson and Fisher (1960) found that the white pine weevil (Pissodes strobi Peck) was repelled by resin, with resin from some tree species being more repellant than others. Bordasch and Berryman (1977) reported that resin produced by Abies grandis (Dougl.) Lindl., in response to a wound, had higher levels of the monoterpenes that were the most repellant to the fir engraver beetle (Scolytus ventralis Lec.). Hanover and Furniss (1966) reported significant differences in cortical oleoresin composition between unattacked, but presumably susceptible Douglas-fir trees and trees that resisted attack by the Douglas-fir bark beetle.

Stroh and Gerhold (1965) found the resin canal system of eastern white pine (Pinus strobus L.) to be important in the resistance of that species to weevils. When the weevil encountered a resin duct while feeding, it would not penetrate the epithelial cells, but would either go around the canal or stop feeding. Bennett (1954) related the susceptibility of several pine species to the pine needle miner (Exoteleia pinifoliella Chamb.) with needle anatomy. Those species with numerous, large resin canals were most resistant to the miner. As with the weevil, the miner appeared to avoid the resin canals when feeding.

Anatomy of Resin Canals

Resin canals are a common feature of the xylem and bark of many conifers, although they have not been described for blue spruce. In white spruce there does not appear to be a functional interconnection between the resin canals in the two tissues (Thomson and Sifton, 1925). There are radial and vertical systems in both of these tissues which also do not appear to interconnect.

The resin canal is a long narrow tube lined with epithelial cells. In white spruce the xylem resin canals may reach 70 cm in length (Bannan, 1936). In transverse section, the xylem resin canal of Pinus pinea L. consists of six or eight epithelial cells surrounding the canal (Wooding and Northcote, 1965). In longitudinal section, the cells are elongated. These cells contain a large number of plastids, which have little internal structure. Each plastid is covered by endoplasmic reticulum. Parenchyma cells adjacent to the ducts have plastids with a normal internal organization.

The resin is produced in the epithelial cells and is secreted into the resin duct cavity. Fahn and Benayoun (1976) suggested that a resin droplet is first surrounded by a plasmalemma invagination, then detaches from the cytoplasm, undergoes lysis, and is released to the apoplast.

Werker and Fahn (1969) described in detail the differentiation of the resin ducts in the xylem of Pinus halepensis Mill. The resin duct initials can be distinguished after the first tangential divisions of the cambium. The initials enlarge and an intercellular space develops between the initials. The space expands and the number of cells

becomes six or more. Fahn and Benayoun (1976) suggest that "Golgi vesicles" carrying lytic enzymes are eliminated from the cytoplasm in the form of multivesicular structures. These structures release enzymes which dissolve the middle lamella where the duct cavity will form schizogenously.

The resin ducts of the needle also develop schizogenously. In Douglas-fir the resin ducts are one of the first tissues to differentiate (Owens, 1968). The resin ducts appear soon after the needle starts to elongate. The differentiation of the resin duct begins just below the needle tip and proceeds basipetally. It does not extend to the petiole nor does it form a continuous resin system with the stem.

The lack of interconnecting systems between needle, xylem, and cortical tissues indicates that their biosynthetic systems are also independent. The genetic implications of this fact are of great interest especially from the standpoint of gene control, terpene metabolism, and the function of oleoresin components in pest resistance.

Genetics of Monoterpenes

Monoterpenes appear to be synthesized from the same basic components through the condensation of isopentenyl pyrophosphate (Nicholas, 1963). The concentration of a specific monoterpene is dependent on which biochemical conversions occur. Therefore, by studying the genetics of monoterpene levels in oleoresin an understanding is gained of the gene control of biosynthetic pathways.

The concentrations of monoterpenes in tree oleoresin systems appear to be under strong genetic control. This is evidenced by the monoterpene composition of clones grown under different site conditions.

For example, clones of loblolly pine (Pinus taeda L.) showed little variation within a clone when planted at different locations (Schmidtling, 1974). Similar results were obtained with western white pine (Pinus monticola Dougl.) (Hanover, 1966a) and Scotch pine (P. sylvestris L.) (Thorin and Nommik, 1974). These and numerous repeatability studies indicate that monoterpene composition is under strong genetic control.

The gene control of terpene compounds has worked out in most detail for species of mint (Mentha spp.) and is reviewed by Hefendehl and Murray (1976). High versus low levels of a monoterpene were found to be controlled by a single gene pair for each monoterpene. Similar genetic control has been shown for certain cortical monoterpenes in trees. Single gene control for 3-carene was shown in western white pine (Hanover, 1966b) and maritime pine (Pinus pinaster Ait.) (Baradat et al., 1972). Myrcene was also found to be controlled by a single gene pair in maritime pine (Baradat et al., 1975). This gene was found to be on the same chromosome as the one that controlled the concentration of 3-carene and the recombination value for the two loci was 0.10. Single gene control was reported in slash pine for the levels of β-pinene, myrcene (Squillace, 1971), limonene, and β-phellandrene (Squillace, 1976). There was no evidence for linkage of these genes. In all cases the allele for the high level of the monoterpene showed dominance.

The levels of monoterpenes present in tree tissues have been used as genetic markers. Squillace (1976) used monoterpenes to estimate the contamination by outside pollen in a seed orchard. Monoterpenes have been widely used for chemosystematic studies. Adams (1977) compared

the use of electrophoretic variants and monoterpenes to distinguish among populations of Juniperus ashei Buchholz. He reported that monoterpenes agreed more closely with the morphological data and gave more meaningful geographic groupings than did electrophoretic variants.

Monoterpenes are very useful for confirming the identity of cultivars. Rottink and Hanover (1972) were able to distinguish among ten blue spruce cultivars on the basis of cortical oleoresin composition. The seed source of Scotch pine Christmas trees could be identified by the composition of the cortical oleoresin (Bridgen et al., 1979). Foliar monoterpenes could be used to distinguish 54 of 55 possible pairs of cultivars of Juniperus horizontalis Moench (Fretz, 1977).

Monoterpenes are also useful in the verification of putative hybrids. Hanover and Wilkinson (1969) used the levels of 3-carene in the parents and in the hybrid to confirm the artificial hybrid between white spruce and blue spruce. The foliar monoterpenes of the Rosendahl spruce (a natural putative hybrid between white spruce and black spruce (Picea mariana (Mill.) B.S.P.)) showed characteristics of both species and some that were intermediate (Von Rudloff and Holst, 1968). However, the hybrid nature of the Rosendahl spruce has been questioned recently (Parker and McLachlan, 1978).

Objectives

Monoterpenes appear to have some biological functions, but in order to understand better the relationship of these substances to tree physiology and to other organisms, it is important to understand monoterpene variation and control of the variation within the tree. Therefore, the overall objective of this research was to describe the

genetics and physiology of the different resin systems in blue spruce. The specific objectives were:

1. To compare the concentrations and composition of the volatile oil of needles, xylem, and bark.
2. To determine the concentrations and composition of monoterpenes in these tissues at different positions within the crown.
3. To compare the concentrations and composition of the volatile oils of needles, stem, and cones in relation to development throughout the growing season.
4. To compare the interrelationships among monoterpenes in these tree organs by correlation analysis.

CHAPTER II

GENERAL METHODOLOGY

The method used to extract terpenes from tree tissues affects not only the quantities of oil, but also the composition of the oil. For example, grinding a leaf sample in the presence of oxygen may result in the formation of the artefact α-hexenal (Major et al., 1963). Von Rudloff (1975a) found this compound to constitute up to 50% of the oil in samples of some conifers at certain times of the year. For this reason, different methods of obtaining oil samples were compared for suitability in subsequent experiments.

Materials and Methods

Needle samples were collected in October 1979 from a single blue spruce tree on the Michigan State University campus. The needles were removed from the branches and divided into eight samples of 10 g each. Five samples of five needles each were also taken from the bulk sample. Four of the 10 g samples were extracted with pentane by homogenizing for 3 minutes in a VirTis '45' homogenizer with sufficient pentane to cover the needles. The extract was filtered and the residue washed with pentane. The extract was then concentrated by evaporation under an air stream to a volume of 5 ml. The other four 10 g samples were extracted by steam distillation in a circulatory steam distillation apparatus modified by Hiltunen (personal communication). The samples were steam distilled for 8 hours after which the distillate was removed.

The apparatus was rinsed twice with pentane, and this rinse was combined with the distillate. The pentane was concentrated under an air stream to a volume of 5 ml. Needles from the five-needle samples were cut into sections approximately 4 mm in length and combined with 50 µl of pentane for 3 to 5 minutes. The pentane solution was then injected into the gas chromatograph.

The effects of different lengths of extraction times were determined by performing steam distillations for varying time periods with a second group of needle samples. Two steam distillations were performed on 10 g samples for each of seven time periods. The extraction times were 2,4,6,8,10,20, and 24 hours.

Monoterpenes in the prepared extracts were assayed by gas-liquid chromatography. Stainless steel columns 6 mm in diameter and 240 cm in length were packed with 7.5% SE-30 on 60/80 mesh Chromasorb W-AW. Analyses were done on a Hewlett-Packard model 5700A gas chromatograph equipped with a flame ionization detector. The injection port and detector temperatures were 300°C. The temperature program was 70°C for 8 min., then increasing 8 /min to a final temperature of 250°C. The helium flow rate was 80 ml/min. Samples of 3 µl were injected. A second column packing of 10% β, β-oxydipropionitrile on 60/80 mesh Chromasorb W-AW was used on a F & M model 700 gas chromatograph equipped with a flame ionization detector. The injection port temperature was 120°C, the detector was 150°C, and the analysis was isothermal at 70°C. Helium flow rate was 30 ml/min. for this column. Samples of 5 µl were injected. Peak areas for both gas chromatographs were determined by a Hewlett-Packard 3370A integrator. Heptane was used as an internal

standard by adding 5 μl heptane/ml pentane. Standard curves of the response of known concentrations of monoterpenes relative to the response of heptane were prepared for quantifying plant monoterpenes.

The needle monoterpenes were identified by gas chromatography-mass spectrometry on an LKB 9000 mass spectrometer. For routine work the peaks were identified by their retention times.

To determine the efficiency of recovery of monoterpenes during extraction, 20 μl of known monoterpenes were added prior to extraction.

Results and Discussion

The effects of differing extraction times on measurement of total monoterpenes is given in figure 3. There was an increase in the amounts of volatile oils with extraction times, except the value for 24 hours was lower than that determined for 20 hours. The composition of the oil also varied with different extraction times. The changes in the amounts and percentages of some of the monoterpenes are given in figures 3 and 4. The percentages of α-pinene, camphene, myrcene, and limonene increased with increasing extraction times whereas the percentages of camphor, borneol, and bornyl acetate decreased with increasing extraction times. Even though the percentages of some of the compounds decreased over time, the amounts of each of the compounds increased for the first 20 hours of extraction, with the exception of camphor which remained constant over all extraction times. After 8 to 10 hours there was little change in the percentages of the compounds. For this reason 8 hour extractions were chosen as the best practical extraction time.

The results of the comparison of the different methods of extraction are given in tables 1 and 2. All three methods produced oils of

Figure 3. Changes in the amounts of foliar monoterpenes associated with length of extraction period.

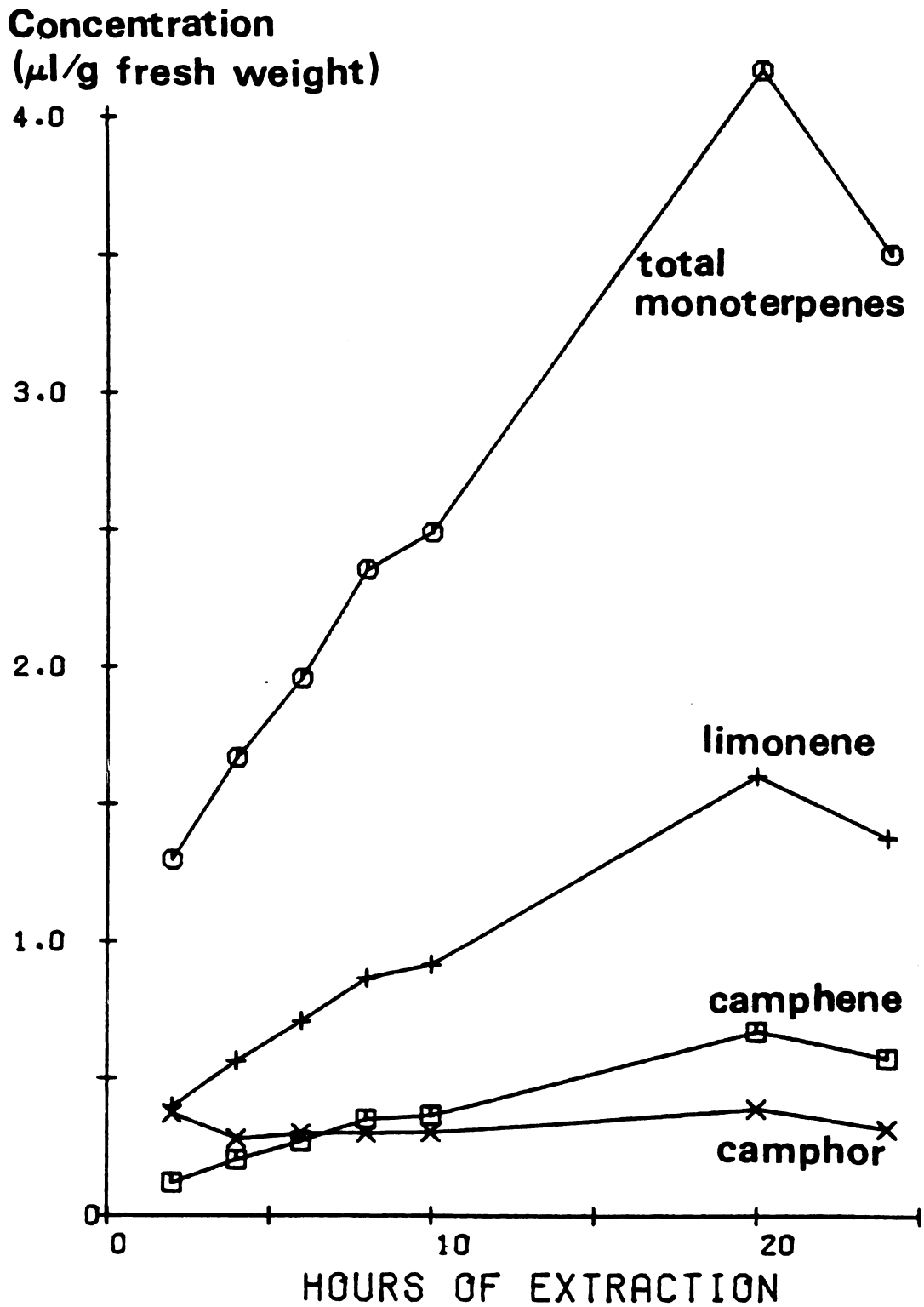

Concentration
(μl/g fresh weight)
4.0
3.0
2.0
1.0
0
total
monoterpenes
limonene
camphene
camphor
0
10
20
HOURS OF EXTRACTION

Figure 3.

Figure 4. Changes in the composition of foliar volatile oil associated with length of extraction period.

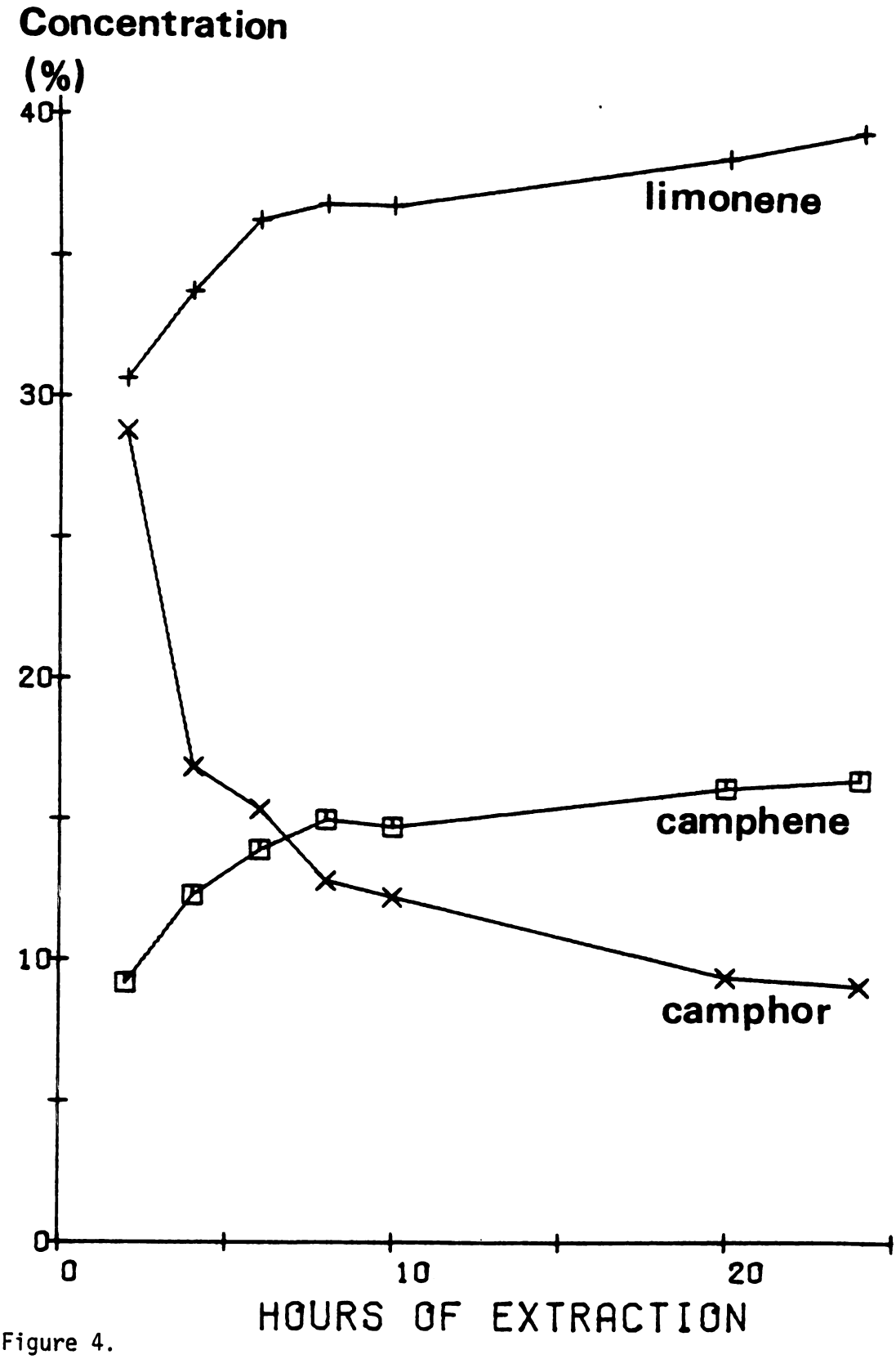

Concentration
(%)
40
30
20
10
0
limonene
camphene
camphor
0
10
20
HOURS OF EXTRACTION

Figure 4.

Table 1.--Mean concentrations of foliar monoterpenes in blue spruce from two methods of extraction based on tissue fresh weight

Monoterpenes	Steam distillation	Pentane extraction
	--- µl monoterpene per g fresh weight---	
Tricyclene	.05 ± 4%[a]	.09 ± 14%
α-pinene	.22 ± 3	.43 ± 8
Camphene	.48 ± 4	.66 ± 8
β-pinene	.01 ± 4	.03 ± 40
Myrcene	.19 ± 6	.29 ± 14
Limonene	.77 ± 3	.98 ± 8
Camphor	.09 ± 20	.09 ± 22
Borneol	.23 ± 5	.24 ± 15
α-terpineol	.03 ± 17	.06 ± 22
Borneol acetate	.69 ± 2	.78 ± 10
Total monoterpenes	2.78 ± 3	3.65 ± 10

[a]Values are mean and standard error in percent of four extractions.

Table 2.--Mean concentration of foliar monoterpenes in blue spruce determined from three methods of extraction

Monoterpenes	Five needle sample	Pentane extraction	Steam distillation
	---percent of total monoterpenes---		
Tricyclene	2.4 ± 0.8%[a]	2.5 ± 4.8%[b]	1.7 ± 1.5%[b]
α-pinene	12.5 ± 1.0	12.0 ± 2.9	8.0 ± 1.2
Camphene	19.2 ± 0.8	18.3 ± 2.4	17.4 ± 1.8
β-pinene	0.5 ± 7.4	0.8 ±31.4	0.4 ± 5.9
Myrcene	7.3 ± 3.7	7.9 ± 4.3	6.8 ± 3.3
Limonene	29.6 ± 2.3	26.5 ± 2.7	27.8 ± 1.0
Camphor	1.4 ±22.4	2.4 ±12.6	3.1 ±17.0
Borneol	6.0 ± 2.8	6.5 ± 5.6	8.4 ± 3.1
α-terpineol	1.0 ± 8.9	1.5 ±12.7	1.0 ±14.0
Bornyl acetate	19.7 ± 1.9	21.4 ± 0.9	25.0 ± 1.9

[a]Values are means and standard errors in percent of five extractions.

[b]Values are means and standard errors in percent of four extractions.

similar composition. The pentane extractions had higher yields than did the steam distillation. The steam distillation extracts yielded an oil with a higher proportion of oxygenated monoterpenes. However, sample reproducibility was better for steam distillations than for pentane extractions whether the levels of the monoterpenes were expressed as quantities of the monoterpenes or as a percentage of the total yield. Because of its superior reproducibility, the steam distillation method was chosen over the pentane extraction method for subsequent work.

The reproducibility of the composition of the oil was similar for the extracts of five needles and the steam distillation extracts. With samples of five needles it would be difficult to determine the amount of oil/g of tissue and this was not attempted. Von Rudloff (1967), was able to analyze the volatiles from a single white spruce needle. He found differences from needle to needle in the yield of oil and recommended a sample of larger size to give a more representative measure of composition.

Losses of monoterpenes during extraction were determined for α-pinene, limonene, and bornyl acetate. Steam distillation resulted in 50.1% recovery of α-pinene, 67.2% of limonene, and 84.4% of bornyl acetate. About 40 to 50% of the amount lost was due to concentrating the extract and the rinses of 50 ml to 5 ml. The remaining losses probably occurred during the distillation process. Because of the differential recovery of the monoterpenes, the monoterpene composition of the extract would not be identical to the monoterpene composition of the tissue. However, seasonal and within-tree variation patterns of the amounts of a monoterpene per gram of tissue would not be affected by these differential losses.

There were no breakdown products observed as a result of steam distillation of the known amounts of monoterpenes. Lack of total recovery of the monoterpenes did not appear to be the result of monoterpene interconversion.

There were large losses of monoterpenes during the distillation processes, but the estimations for a sample were highly repeatable. Therefore, steam distillation was judged to be the best technique to employ for monoterpene analyses.

CHAPTER III

WITHIN-TREE VARIATION IN VOLATILE OILS

For chemosystematic purposes and to better understand mechanisms of host resistance to insects, diseases, and animal browsing, knowledge of the variation of monoterpenes within a tree is essential. Similar monoterpene compositions were found in needles and cortical oleoresin in western white pine (Hanover, 1972) and grand fir (Von Rudloff, 1976). In Scotch pine, the composition of needle, xylem, and phloem or cortex oil was found to differ (Tobolski, 1968; Hiltunen, 1976). Roberts (1970) found needle and branch cortex oil to be similar to each other, but different from xylem oil in slash pine. Zavarin et al. (1971b) reported differences between the monoterpene composition of cortical and phloem oleoresin in subalpine fir (Abies lasiocarpa (Hook) Nutt.). Von Rudloff (1972, 1975b and c) found large differences between needle and stem composition of white spruce, black spruce, and blue spruce.

The terpene composition at different positions in the tree may also be important. No differences in monoterpene composition with height were found for xylem oleoresin in Pinus muricata D. Don and P. radiata D. Don (Blight and McDonald, 1964), ponderosa pine (Smith, 1964), and loblolly pine (Rockwood, 1973b). Significant differences in xylem oleoresin composition do occur with height in slash pine (Franklin, 1976; Roberts, 1970) and for four Abies species (Zavarin, 1968). Rockwood (1973b) found minimal variation with height for cortical monoterpenes in slash pine. No significant differences in the

composition of needle monoterpenes with height were found in western white pine (Hunt and Von Rudloff, 1977), western hemlock Tsuga heterophylla (Raf.) Sarg.) (Von Rudloff, 1975d), two Abies species (Hunt and Von Rudloff, 1974), white spruce, or black spruce (Von Rudloff, 1967). However, differences with height were found for a single white spruce tree (Von Rudloff, 1967) and for Engelmann spruce (Picea engelmannii Parry) (Ogilvie and Von Rudloff, 1968).

The monoterpene composition may also vary with the age of the tissue sampled. Hanover (1966a), Tobolski (1968), Roberts (1970), and Bernard-Dagan et al. (1971) all found differences in cortical oleoresin composition in resin samples from tissues of different ages in several species of pines.

Materials and Methods

Variation among tissues of the same age

Branches were collected from the top, middle, and bottom of eight blue spruce trees approximately 10 meters in height on the Michigan State University campus on July 11, 1978. Only the fully mature 1977 growth was analyzed. The branches were dipped into liquid nitrogen which greatly facilitated needle removal. The stem was separated into xylem and bark. All samples were stored at -20°C until analyzed. Sample weights varied from 11.5 to 43.3 g for needles, 1.1 to 12.0 g for xylem, and 2.4 to 22.0 g for bark. Stem samples were cut into sections 1 cm in length prior to distillation. All samples were steam distilled for 8 hours and the oils analyzed by gas-liquid chromatography the same day. Distillation and gas chromatographic conditions were the same as described in chapter 2. The samples were concentrated to 5 ml

except for the xylem samples which were taken to 1 ml. A duplicate sample was collected for each sample analyzed. The second sample was oven dried at 70°C until a constant weight was obtained. The dry weight/fresh weight ratio was calculated. This factor was used to convert the yield of monoterpenes into µl/g dry weight of tissue.

The proportion of resin canals to other tissues was determined with a Wild stereomicroscope, model M5 with a measuring eyepiece. Samples were collected from the top and bottom of four trees sampled for monoterpenes. The cross sectional area of xylem, bark, and the resin canals in each were determined from free hand stem sections. Needle samples of five needles each were collected from the top and bottom of two trees. Free hand cross-sections were made at 1 mm intervals throughout the length of each needle. The volume of the needles and the volume of the resin canals were determined.

Variation among tree organs of different ages

Four six-year-old blue spruce trees approximately 1 meter in height were dug from the nursery beds at the Tree Research Center at Michigan State University in November 1978. Each tree was divided into four portions: the stem portion of the 1978 growth of the uppermost whorl, the 1976 internode of the main stem, the basal 15 cm of the main stem, and root samples. Needles were removed after dipping the branches into liquid nitrogen. Twelve samples were collected from each tree including three from each position in the tree. Two of the three samples from each tree were steam distilled and analyzed and one was used to determine the dry weight/fresh weight ratio. The samples ranged from 5.3 to 23.9 g in weight. Steam distillation was performed

as previously described.

Statistical analysis of percentage data in each of the studies was performed after arcsine transformation.

Results

Among tissue variation

The amounts of volatiles recovered differed greatly among tissues (table 3). The bark had an average of almost three times that found in the needles and eleven times that found in the xylem. There were few qualitative differences among the tissues. The xylem extracts were comparatively more simple than those of the other tissues, being composed primarily of α- and β-pinene. Although the major components of the root samples were also α- and β-pinene (table 4), they had a different composition than the stem xylem. The roots had higher yields of oil and had a larger proportion of compounds other than α- and β-pinene than did xylem. Santene was present in the volatile oil of the roots, but not in the xylem. The needle extracts contained a greater number of monoterpene compounds than did the other tissues. The compounds tricyclene, 1:8 cineole, camphor, and borneol were present in appreciable amounts only in the needles. Gamma-terpinene was only present in appreciable amounts in bark extracts. The bark extracts contained several unidentified high molecular weight compounds, most of which differed significantly in concentration among trees (table 5). These unknown compounds were not detected, or were present in very small amounts, in the other tissues.

There were many quantitative differences among tissues. The needle extracts had a higher proportion of the oxygenated monoterpenes,

Table 3.--Mean volatile oil composition over all positions in crown in different tissues of blue spruce

Monoterpene	Tissue		
	Xylem	Foliage	Bark
	---percent of total monoterpenes[a]---		
Santene	--	.2	.1
Tricyclene	--	.7	--
α-pinene	71.4	8.3	31.7
Camphene	1.0	12.6	1.2
β-pinene	20.0	.9	6.0
Sabinene	2.4	--	1.7
Myrcene	1.5	9.8	7.1
3-carene	.4	.5	22.2
Limonene	3.6	30.9	19.5
β-phellandrene	--	1.0	1.4
γ-terpinene	--	--	1.0
1:8 cineole	--	.8	--
Terpinolene	--	.8	4.3
Camphor	--	11.2	--
Borneol	--	3.5	--
Terpinen-4-ol	--	.9	1.0
α-terpineol	--	2.8	.6
Bornyl acetate	--	14.0	2.6
	---μl/g dry weight[a]---		
Total unknown compounds	--	--	1.83
Total monoterpenes	.62	2.38	6.89

[a]Means are of eight trees averaged over three positions in the crown.

Table 4.--Variation in the monoterpene composition in stems and roots with position in blue spruce one meter in height

Monoterpenes	Position in the tree			
	Top	Middle	Bottom	Roots
	---Percent of total monoterpenes---			
Santene	.00 a[1]	.00 a	.04 b	1.50 c
α-pinene	43.0 a	61.6 b	64.7 c	65.9 d
Camphene	1.1 a	1.4 b	1.4 b	2.4 c
β-pinene	8.0 a	12.2 b	14.7 c	17.2 d
Sabinene	2.2 a	1.3 b	2.2 a	2.1 a
Myrcene	4.0 a	2.8 b	2.6 c	2.1 d
3-carene	18.5 a	5.1 b	1.2 d	1.4 c
Limonene	14.6 a	9.5 b	6.8 c	2.2 d
β-phellandrene	1.2 a	1.0 a	1.0 a	1.1 a
γ-terpinene	.4 a	.0 a	.0 a	.0 a
Terpinolene	3.3 a	1.2 d	1.5 c	1.8 b
Bornyl acetate	1.7 a	2.1 b	1.8 c	.2 d

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Table 5.--Analysis of within and among tree variation in bark monoterpenes of blue spruce

Monoterpene	Monoterpene composition as µl/g dry weight		Monoterpene composition as percent total monoterpene	
	F-value due to		F-value due to	
	Tree	Position	Tree	Position
Santene	4.07 *[1]	7.74 **	1.66	5.67 **
α-pinene	9.76 **	3.12	30.44 **	3.59
Camphene	7.14 **	1.46	19.47 **	4.75 *
β-pinene	14.13 **	0.14	46.99 **	2.65
Sabinene	3.19 *	2.32	4.34 **	0.83
Myrcene	5.19 **	0.97	27.85 **	0.31
3-carene	7.42 **	1.69	7.93 **	1.29
Limonene	1.76	2.02	29.85 **	1.43
β-phellandrene	1.05	1.15	1.07	0.56
γ-terpinene	3.43 *	3.65	5.14 **	3.70
Terpinolene	5.03 **	3.68	3.93 *	1.16
Terpinen-4-ol	1.66	4.90 *	1.04	4.04 *
α-terpineol	1.61	2.71	1.08	3.04
Bornyl acetate	11.69 **	5.75 *	33.01 **	10.42 **
Unknown 19	22.36 **	6.84 **	--	--
Unknown 20	3.01 *	1.43	--	--
Unknown 23	5.03 **	2.24	--	--
Unknown 25	3.84 *	1.06	--	--
Unknown 26	2.31	1.73	--	--
Unknown 27	7.06 **	0.74	--	--
Total monoterpenes	4.56 **	2.78	--	--

[1]Values followed by * and ** are significant at the five and one percent level respectively.

camphor, borneol, α-terpineol, and bornyl acetate than the other tissues. Needles also had a higher proportion of limonene and camphene. Alpha-pinene and β-pinene were proportionally higher in the bark than in the needles. Three-carene was present in large amounts in the bark samples and present in low amounts in the other tissues.

Variation in xylem monoterpenes

Large differences were found among trees in the amount of total monoterpenes in the xylem (table 6). The tree with the lowest average yield of monoterpenes over the three crown positions had only 0.14 µl/g dry weight and the tree with the highest yield had 0.86 µl/g dry weight. There were significant differences among trees in the amounts of α-pinene, β-pinene, sabinene, and limonene (table 6). When the composition was expressed as a percentage of the total monoterpene fraction, the same compounds, with the exception of limonene, differed among trees.

There were differences in the total yield of monoterpenes from different heights (tables 6 and 7). There were higher amounts in the xylem at the top of the tree than at the base. All of the monoterpenes except 3-carene differed significantly in concentration with position in the crown. However, when the data were expressed as a percentage of the total monoterpenes only sabinene, myrcene, and limonene differed with position in the crown (tables 6 and 8).

Variation in needle monoterpenes

Large differences among trees were found for the total needle monoterpenes. The tree with the highest monoterpene levels had three times that with the lowest levels. There were statistically significant differences among trees for the amounts of most monoterpenes

Table 6.--Analysis of within and among tree variation in xylem monoterpenes of blue spruce

Monoterpene	Monoterpene composition as µl/g dry weight		Monoterpene composition as percent total monoterpene	
	F-value due to		F-value due to	
	Tree	Position	Tree	Position
α-pinene	5.79 **[a]	26.08 **	3.94 *	0.27
Camphene	1.33	17.65 **	0.21	2.53
β-pinene	21.96 **	14.38 **	3.94	0.27
Sabinene	3.26 *	8.30 **	2.66	5.26 *
Myrcene	2.06	17.76 **	2.60	13.38 **
3-carene	0.74	4.02 *	0.62	3.85 *
Limonene	2.96 *	22.97 **	1.08	9.58 **
Total monoterpenes	5.99 **	31.64 **	--	--

[a]Values followed by * and ** are significant at the five and one percent level respectively.

Table 7.--Variation in concentration of xylem monoterpenes at different positions in the crown

Monoterpene	Position in the crown		
	Top	Middle	Bottom
	---µl/g dry weight---		
α-pinene	.68 a[1]	.41 b	.23 c
Camphene	.012 a	.004 b	.003 b
β-pinene	.18 a	.11 b	.08 b
Sabinene	.029 a	.011 b	.005 b
Myrcene	.021 a	.004 b	.002 b
3-carene	.006 a	.000 a	.001 a
Limonene	.045 a	.018 b	.004 c
Total monoterpenes	.98 a	.55 b	.32 c

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Table 8.--Variation in proportions of xylem monoterpenes at different positions in the crown

Monoterpene	Position in the crown		
	Top	Middle	Bottom
	---percent of total monoterpenes---		
α-pinene	69.5 a[1]	74.3 a	81.1 a
Camphene	1.4 a	0.5 b	0.4 b
β-pinene	17.2 a	19.5 a	15.8 a
Sabinene	2.8 a	1.4 ab	0.4 b
Myrcene	1.6 a	0.1 b	0.0 b
3-carene	0.4 a	0.0 b	0.0 b
Limonene	4.6 a	1.6 b	0.2 c

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

when expressed as μl/g of dry weight tissue (table 9). Most of the compounds were also significantly different among trees when the data was expressed as a percentage of the total monoterpenes.

There were also differences in the amounts of total monoterpenes in needles from different positions in the crown (tables 9 and 10). There was little difference between the amounts in the top and middle of the crown, but there was almost twice as much volatile oil in needles from the base of the tree. Fewer compounds showed significant differences with crown position, when the data were expressed as a percentage of the total monoterpenes (tables 9 and 11).

Variation in bark monoterpenes

The yield of monoterpenes from the bark was significantly different among trees. The amounts averaged over the three crown positions ranged from 4.42 to 9.96 μl/g of dry weight tissue. There were significant differences among trees for both the amounts and the percentages of almost every compound (table 5). In contrast to the other tissues, the total yield of monoterpenes in the bark did not differ significantly with position in the crown (tables 5 and 12). Only santene, terpinen-4-ol, bornyl acetate, and unknown 19 differed significantly in amount with position in the crown. When the amounts are expressed as a percentage of the total monoterpenes, the same monoterpenes show significant differences with height. In addition, camphene and α-pinene were significantly different with height when the data was expressed in this form (tables 5 and 13).

Table 9.--Analysis of within and among tree variation in foliar monoterpenes of blue spruce

Monoterpene	Monoterpene composition as µl/g dry weight		Monoterpene composition as percent total monoterpene	
	F-value due to		F-value due to	
	Tree	Position	Tree	Position
Santene	15.01 **	3.82 *	31.57 **	18.61 **
Tricyclene	2.46	6.89 **	8.57 **	0.26
α-pinene	6.05 **	13.45 **	4.98 **	11.77 **
Camphene	6.27 **	17.65 **	14.62 **	11.73 **
β-pinene	12.54 **	6.62 **	12.16 **	16.23 **
Sabinene	1.83	2.91	2.31	3.00
Myrcene	17.07 **	19.02 **	43.72 **	0.45
3-carene	2.13	2.78	1.39	4.77 *
Limonene	11.36 **	30.78 **	55.94 **	4.08 *
β-phellandrene	14.39 **	6.12 *	7.32 **	1.01
1:8 cineole	3.24 *	9.81 **	2.65	2.33
Terpinolene	3.99 *	9.45 **	0.47	0.36
Camphor	14.55 **	22.35 **	29.81 **	10.00 **
Borneol	25.18 **	102.95 **	5.14 **	3.56
Terpinen-4-ol	3.74 *	1.80	2.04	8.21 **
α-terpineol	23.99 **	38.88 **	2.78 *	0.86
Bornyl acetate	5.88 **	9.58 **	150.15 **	10.94 **
Total monoterpenes	8.32 **	30.75 **	--	--

[a]Values followed by * and ** are significant at five and one percent respectively.

Table 10.--Variation in concentrations of blue spruce foliar monoterpenes at different positions in the crown

Monoterpene	Position in the crown		
	Top	Middle	Bottom
	---µl/g dry weight---		
Santene	.0047 a[1]	.0054 ab	.0065 b
Tricyclene	.012 a	.013 a	.026 b
α-pinene	.16 a	.17 a	.26 b
Camphene	.26 a	.28 a	.46 b
β-pinene	.019 a	.018 a	.026 b
Sabinene	.0024 a	.0003 a	.0012 a
Myrcene	.19 a	.18 a	.33 b
3-carene	.018 a	.008 a	.007 a
Limonene	.53 a	.59 a	1.09 b
β-phellandrene	.019 a	.016 a	.033 b
1:8 cineole	.013 a	.011 a	.033 b
Terpinolene	.016 a	.017 a	.026 b
Camphor	.19 a	.18 a	.43 b
Borneol	.057 a	.071 b	.121 c
Terpinen-4-ol	.021 a	.019 a	.023 a
α-terpineol	.055 a	.052 a	.091 b
Bornyl acetate	.22 a	.23 a	.55 b
Total	1.78 a	1.86 a	3.51 b

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Table 11.--Variation in proportions of blue spruce foliar monoterpenes at different positions in the crown

Monoterpene	Position in the crown		
	Top	Middle	Bottom
	---Percent of total monoterpenes---		
Santene	.30 a[1]	.32 a	.18 b
Tricyclene	.8 a	.8 a	.7 a
α-pinene	9.3 a	9.3 a	7.5 a
Camphene	14.8 a	15.1 a	13.2 b
β-pinene	1.06 a	.99 b	.72 c
Sabinene	.1 a	.0 a	.0 a
3-carene	.7 a	.2 b	.1 b
Myrcene	10.2 a	10.0 a	9.6 a
Limonene	28.6 a	30.4 b	30.7 b
β-phellandrene	.9 a	.6 a	.7 a
1:8 cineole	.6 a	.4 a	.9 a
Terpinolene	.8 a	.9 a	.8 a
Camphor	8.9 a	8.5 a	11.5 b
Borneol	3.2 a	4.0 a	3.6 a
Terpinen-4-ol	1.3 a	1.1 b	.7 c
α-terpineol	3.1 a	2.7 a	2.6 a
Bornyl acetate	12.5 a	11.9 a	14.2 b

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Table 12.--Variation in concentrations of bark monoterpenes at different positions in the crown.

Monoterpene	Position in the crown: Top		Middle		Bottom	
	---µl/g dry weight---					
Santene	.004	a[1]	.005	a	.009	b
α-pinene	2.63	a	2.00	a	1.92	a
Camphene	.10	a	.07	a	.08	a
β-pinene	.41	a	.40	a	.43	a
Sabinene	.14	a	.12	a	.10	a
Myrcene	.53	a	.48	a	.45	a
3-carene	1.67	a	1.51	a	1.41	a
Limonene	1.49	a	1.34	a	1.20	a
β-phellandrene	.11	a	.10	a	.08	a
γ-terpinene	.07	a	.07	a	.06	a
Terpinolene	.34	a	.30	a	.25	a
Terpinen-4-ol	.09	a	.07	ab	.04	b
α-terpineol	.04	a	.05	a	.03	a
Bornyl acetate	.24	a	.16	b	.13	b
Unknown 19	.15	a	.11	b	.10	b
Unknown 20	.27	a	.31	a	.25	a
Unknown 23	1.03	a	1.16	a	0.91	a
Unknown 25	.19	a	.23	a	.21	a
Unknown 26	.11	a	.13	a	.12	a
Unknown 27	.08	a	.07	a	.07	a
Total monoterpenes	7.84	a	6.67	a	6.17	a

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Table 13.--Variation in proportions of bark monoterpenes at different positions in the crown

Monoterpene	Position in the crown		
	Top	Middle	Bottom
	---Percent of total monoterpenes---		
Santene	.04 a[1]	.07 a	.14 b
α-pinene	31.6 a	28.5 a	30.3 a
Camphene	1.05 ab	.94 a	1.19 b
β-pinene	5.3 a	5.7 a	6.3 a
Sabinene	1.9 a	1.7 a	1.6 a
Myrcene	6.9 a	7.2 a	7.1 a
3-carene	21.3 a	22.8 a	23.1 a
Limonene	20.0 a	20.7 a	19.4 a
β-phellandrene	1.4 a	1.6 a	1.3 a
γ-terpinene	1.0 a	1.0 a	.9 a
Terpinolene	4.3 a	4.5 a	4.1 a
Terpinen-4-ol	1.1 a	1.0 a	.5 b
α-terpineol	.5 a	.7 a	.6 a
Bornyl acetate	2.6 a	2.1 b	1.9 c

[1]Means across columns followed by the same letter are not significantly different at the 5 percent level.

Anatomy of resin canals

There is a possibility that the large within-tree variation and among-tree differences in the yield of total monoterpenes might have an anatomical basis. Therefore, the ratio of resin canals to other tissues was determined. These fractions were then used in correlation analyses with the total monoterpene yield.

The correlation between the proportion of resin canals to other tissues and the yield of monoterpenes was highly significant for the xylem ($r = +.89$, $p \leq .01$). There was no difference between the top and bottom of the tree in the average area of an individual resin canal in the xylem ($F = 0.5$).

The needles had resin canals along two edges of the needle and they did not appear to be continuous over the entire length of the needle. A resin canal would appear to end at one point, then begin further along the length of the needle. The ratio of resin canals to other tissues in the needle was highly significantly correlated with the yield of total monoterpenes ($r = +.995$, $p \leq .01$).

There were significant differences in the volume of the resin canals between the top and bottom of a given tree (table 14). These differences were apparent in all portions of the needle. When the distribution of the resin canal within the needle was examined (table 15) it was found that the differences were not simply a uniform increase in the size of the resin canal over the entire length of the needle. The needles from the base of the tree had a larger proportion of resin canals in their tips. The resin canal volume in needles from the base of the tree had 3 and 6.6 times more volume than needles from the top

Table 14.--Variation in the resin canal volume in needles from different positions in the crown of two blue spruce trees

Portion of the needle	Tree 83			Tree 88		
	Position in crown		t value for the difference of means	Position in crown		t value for the difference of means
	Top	Bottom		Top	Bottom	
	$mm^3 \times 10^4$			$mm^3 \times 10^4$		
Entire needle	306	908	7.97 **[a]	427	2807	10.61 **[a]
Base to 1/4 of length from base	143	471	3.81 **	214	1208	13.38 **
1/4 to 1/2 of length from base	106	184	3.32 **	169	923	4.71 **
1/2 to 3/4 of length from base	54	162	4.67 **	40	393	2.67 **
3/4 of length from base to tip	2	90	7.19 **	2	525	5.65 **

[a]Values by ** are significant at the one percent level.

Table 15.--Variation in the percentage of the total resin canal volume in portion of the needle

Portion of the needle	Tree 83			Tree 88		
	Position in crown		t value for the difference of means	Position in crown		t value for the difference of means
	Top	Bottom		Top	Bottom	
	percent[a]			percent[a]		
Base to 1/4 of length from base	45	49	0.84[b]	51	35	1.34
1/4 to 1/2 of length from base	37	21	2.06	41	33	0.67
1/2 to 3/4 of length from base	18	20	0.46	8	14	0.95
3/4 of length from base to tip	0.4	10	6.34 **	0.6	18	8.14 **

[a]Percent calculated by $\% = \frac{(mm^3 \text{ of resin canal portion})}{(mm^3 \text{ of resin canal in needle})} \times 100$

[b]Values followed by ** are significant at the one percent level.

of trees 83 and 88. The canal volumes of the distal 25% of the needles for the same trees were 45 and 263 times greater in needles from the bottom than from the tops.

The correlation between the proportion of resin canal in the bark and the total yield of monoterpenes was lower than for needle and xylem ($r = +.571$, $p \leq .10$).

Variation associated with tree organs of different ages

Large differences were found for the amounts of total monoterpenes both among trees and among positions within the trees (tables 16 and 17). The only monoterpene that did not show significant differences among trees for any analyses was γ-terpinene. There were large differences for the amounts of most individual monoterpenes associated with position in the tree (tables 16 and 17). There was significantly more of all of the compounds, except santene, in samples from the top whorl. Santene was not detected in the samples taken from the upper parts of the tree, but was present in small amounts at the base of the tree, and in larger amounts in the roots. The amounts found in one sample of predominantly fine roots had still higher levels of santene.

The composition of the oil differed within and among trees as well as did the amounts (tables 18 and 4). There were large differences in the composition of the oil from the youngest stem tissue and that from stems two years older. There were smaller differences between samples taken from the base of the tree and those taken from the middle of the tree. The roots differed significantly from the rest of the tree for most of the compounds.

Table 16.--Analysis of variation and covariation in concentrations of monoterpenes in blue spruce trees one meter in height expressed as µl/g dry weight

Monoterpene	F value from analysis of variance for differences among		F value from analysis of covariance for differences among	
	Trees	Position	Trees	Position
Santene	6.99 **[a]	148.30 **	3.53	81.24 **
α-pinene	16.24 **	45.30 **	15.81 **	11.15 **
Camphene	5.11 *	36.02 **	6.57 *	3.96 *
β-pinene	95.79 **	29.42 **	89.95 **	10.81 **
Sabinene	26.62 **	62.58 **	9.44 **	2.53
Myrcene	22.20 **	262.79 **	18.08 **	28.72 **
3-carene	38.58 **	623.99 **	32.71 **	48.01 **
Limonene	11.19 **	163.00 **	8.25 **	25.76 **
β-phellandrene	21.88 **	73.51 **	39.35 **	19.70 **
γ-terpinene	2.28	15.55 **	2.10	1.42
Terpinolene	60.41 **	538.46 **	35.10 **	32.85 **
Bornyl acetate	38.77 **	110.34 **	50.40 **	62.16 **
Total monoterpenes	7.50 **	120.21 **	6.06 **	17.12 **
Total unknown compounds	9.65 **	28.27 **	10.80 **	13.67 **

[a]Values followed by * and ** are significant at the five and one percent levels, respectively.

Table 17.--Variation in concentrations of monoterpenes with height in blue spruce trees one meter in height

Monoterpenes	Position in the tree			
	Top	Middle	Bottom	Roots
	---µl/g dry weight---			
Santene	.000 a[1]	.000 a	.003 a	.027 b
α-pinene	4.33 a	2.75 b	2.16 c	1.13 d
Camphene	.111 a	.064 b	.045 c	.043 c
β-pinene	.838 a	.605 b	.514 b	.329 c
Sabinene	.225 a	.081 b	.065 b	.040 b
Myrcene	.414 a	.133 b	.087 c	.037 d
3-carene	1.878 a	.254 b	.039 c	.025 c
Limonene	1.498 a	.427 b	.236 c	.039 d
β-phellandrene	.123 a	.049 b	.034 bc	.021 c
γ-terpinene	.071 a	.000 b	.000 b	.000 b
Terpinolene	.346 a	.056 b	.053 b	.032 c
Bornyl acetate	.164 a	.098 b	.064 c	.008 d
Total monoterpenes	10.02 a	4.50 b	3.32 c	1.73 d
Total unknown compounds	2.54 a	1.84 b	1.67 b	.78 c

[1]Means across columns followed by the same letter are not significantly different and the 5 percent level.

Table 18.--Analysis of variation and covariation in concentrations of monoterpenes in blue spruce trees one meter in height expressed as a percent of the total monoterpenes

Monoterpene	F value from analysis of variance for differences among: Trees	Position	F value from analysis of covariance for differences among: Trees	Position
Santene	6.30 **[a]	194.87 **	3.55	149.38 **
α-pinene	1190.16 **	586.51 **	1024.10 **	159.18 **
Camphene	5.55 *	63.40 **	3.76	40.72 **
β-pinene	2361.73 **	331.36 **	2255.52 **	36.29 **
Sabinene	23.04 **	8.45 **	9.45 **	9.98 **
Myrcene	57.70 **	93.43 **	57.54 **	29.65 **
3-carene	152.11 **	1499.30 **	146.40 **	273.11 **
Limonene	3.13	480.89 **	2.90	233.77 **
β-phellandrene	56.74 **	1.20	55.44 **	1.63
γ-terpinene	1.63	12.16 **	1.90	1.80
Terpinolene	11.49 **	85.69 **	5.25 *	20.34 **
Bornyl acetate	203.27 **	261.80 **	203.27 **	235.34 **

[a]Values followed by * and ** are significant at the five and one percent levels.

One of the samples analyzed was composed almost entirely of fine roots, 1 mm or less in diameter, in contrast to other root samples from the same tree which contained roots up to 1.5 cm in diameter. Because this was a single sample from one tree, it was not included in any of the statistical analyses. This sample had higher amounts of total monoterpenes and higher percentages of santene, camphene, and 3-carene than did larger roots from the same tree.

Discussion

The results of the study agree with those of previous related work. Von Rudloff (1975c) reported a yield of 2.53 µl of total monoterpenes/g dry weight of needle for a single blue spruce tree. This value is close to the mean of the eight trees in this study. Also, the composition in this study was very similar to that found by Von Rudloff. He reported the presence of linalool, citronellol, and piperitone. These compounds were detected in this study in one sample that was analyzed by gas chromatography-mass spectrometry. The values for these compounds were not included in the analyses because the compounds were absent or present in small amounts. Von Rudloff reported the presence of camphene hydrate also. Camphene hydrate was detected in some samples, but it was very difficult to separate camphene hydrate from camphor, so the two were reported as a single compound. Von Rudloff did not separate the twig into bark and xylem. However, the composition of his twig oil agrees with the composition of our bark extracts. He did not report the presence of large amounts of the high molecular weight compounds which were discovered in this study.

The results of the study indicate that factors which control the monoterpene levels in the tree alter the biosyntheses in the different tissues. The amounts of the total monoterpene fraction increased with height in the crown for the xylem, decreased with height in needles, and showed no consistant pattern for the bark. There are also no general trends across species in the changes in monoterpene composition from one tissue to another. In blue spruce, 3-carene is predominantly found in the bark and is present in minimal amounts in needle and xylem samples. Tobolski (1968) and Hiltunen (1976) found different results for 3-carene in Scotch pine. They found 8.6% and 21%, respectively, in needles and 24% and 39% in xylem.

The low levels of 3-carene in needles and xylem, would explain the discrepancy found by Hanover (1972) between the high 3-carene levels found in cortical oleoresin of blue spruce and the low levels given off to the atmosphere by the tree.

The differences among trees and with position in the tree for total monoterpenes indicate differences in the biosynthesis of monoterpenes, but may not be the result of changes in the biosynthetic pathways. However, changes in the composition of the oil do indicate a change in biosynthetic pathways. Changes in the percentages of several monoterpenes may result from a single change in the biosynthetic pathways that alters the concentrations of one monoterpene. The percentages of all of the remaining monoterpenes would change when the amount of one of the components changes. Measuring only the composition and not the concentrations of the monoterpenes would not allow detection of the large among-tree and within-tree differences found in this study. These facts have important implications to investigators seeking

mechanisms for resistance to insects, pathogens, and animal browsing. The concentrations of monoterpenes, the relative composition of the oil, and tissue specificity may all be important considerations which have often not been accounted for in past studies.

Camphor and bornyl acetate, which are oxygenated monoterpenes, comprised a higher proportion of the volatile oil of the needles from the base of the tree. Terpinen-4-ol and the other monoterpenes which showed significant differences comprised a lower proportion of the oil in needles from the base of the tree. The boiling point of α-pinene is 156.2°C and that of bornyl acetate is 223-224°C. The levels of α-pinene increased with height in the crown, and bornyl acetate decreased, so the pattern of variation cannot be explained on the basis of their volatility.

The finding that the resin canals of the needles were not continuous throughout the entire length of the needle agrees with observations in some other species. Werker and Fahn (1969) found discontinuities in the resin canals in Pinus halepensis. Hendrickson and Lotan (1971) found variation in the number of resin canals in whitebark pine (Pinus albicaulis Engelm.) and limber pine (P. flexilis James) needles. They observed that the number of resin canals was dependent on the position in the needle that the count was taken. The resin canals of Douglas-fir, however, appear to be continuous (Owens, 1968).

The results of comparison of needles from the top and the bottom of the tree indicated that there is a change both in the volume of the resin canals and in their distribution within the needle. This change in the distribution of the resin canals may have an impact on insect behavior, particularly because some insects, such as the pine needle

miner, appear to avoid resin canals when feeding (Bennett, 1954).

Differences in monoterpene distribution within a needle have been noted in other species. Bernard-Dagan et al. (1979) reported that the monoterpene synthesis was not uniform over the entire needle of maritime pine. They found preferential synthesis of monoterpenes in the basal portion of the needle, with the middle and tip of the needle having 16 and 66 times as much 14_C incorporation into monoterpenes as in the base. In contrast sesquiterpenes were synthesized over the entire needle.

Schroger (1916) found that the yield of oil among species was related to the number and size of the resin canals in the needles. The same situation was found within a single species in this study, but on a more quantitative basis. The ratios of resin canals to other tissues in needles and xylem were highly correlated with the yield of volatile oil. Xylem samples from the top of the tree, which had larger amounts of monoterpenes had an increased density of resin canals, rather than increased sizes of resin canals. This increase in density of resin canals could be a response to wind. In Pinus halepensis xylem there was an increase in the number of resin canals in two-year-old trees exposed to wind compared to trees protected from the wind (Fahn and Zamski, 1970). The lower correlation between yield of monoterpenes and the proportion of resin canals in the bark could be due to the presence of more branch to branch variation than in the needles or xylem. The difference could also be due to the presence of the unknown compounds in the bark that were insignificant in needles and xylem. These compounds could take up some volume in the resin canals and introduce more variation.

The differences in concentrations of the monoterpenes in samples from different positions on the stem of small trees could result from several factors. One factor could be the effect of position in the tree on availability of substrates, pH, or other cellular conditions. However, the analyses of the bark samples of the same age from different positions in the large trees showed small differences associated with position in the crown. The samples from the different stem positions in the small trees vary in their proportions of xylem and bark which have greatly different chemical compositions. Therefore, an analysis of covariance was performed using the percentage of xylem in the sample as a covariate. The result was that percent xylem was significantly correlated with the levels of all of the monoterpenes. However, only for the yield of sabinene and the percentage of γ-terpinene was a statistically significant difference for position, as determined by analysis of variance, not significant with analysis of covariance. This would indicate that the chemical differences may be due to more than varying proportions of tissues. The results of this study agree with those found in studies of oleoresin from the cortex of tissues of different ages. Hanover (1966a) found more myrcene and 3-carene in current-year tissue of western white pine compared with older tissues. He also found lower levels (measured on a percent oleoresin basis) of α-pinene and β-pinene in young tissue. Roberts (1970) found an increase in α-pinene and a decrease in myrcene with increasing age in slash pine. Bernard-Dagan et al. (1971) found higher levels of 3-carene, myrcene, and limonene in younger tissues of maritime pine. These results agree with this study when the monoterpene levels are expressed as a

percentage of the total monoterpenes.

Conclusions

The results of these studies have strong implications for chemosystematic work and especially for studies of the relationship between monoterpenes and tree resistance to pests. There are substantial differences among tissues and among tissues of different ages, both in the amounts of resin and in its composition. Tissues must be separated and the appropriate tissue analyzed. For studies of resistance mechanisms the tissue that is attacked by the organism should be analyzed. The within-tree variation detected necessitates the analysis of the tissue in the portion of the crown that is attacked.

In addition to the composition of the oil, the large differences found in the total amounts of the oil among and within trees may also be important in pest resistance mechanisms. In other words, resistance to an insect or a fungus could result from both the amount of oil present in a tissue and its composition. Finally, much attention is now being directed toward volatile emissions from plants of which internal composition is a major determinant.

Amounts and compositions of volatile oils in needles and bark varied sharply from tree to tree, whether the monoterpenes were expressed in absolute amounts or as a percentage of the total monoterpenes. Both bark and needle monoterpenes could be used very effectively in chemosystematic studies. The bark monoterpenes may be better suited to some studies, because of the lack of variability in concentration with height.

Non-genetic factors, such as position in the crown and age of the tissue must be taken into consideration in planning genetic studies. Standardization of the tissue and the position sampled is required for meaningful genetic and chemosystematic work.

The factors that determine the large variation with position in the crown and the age of the tissue are yet to be determined. The causes could be changes that alter the biosynthesis, losses, or conversions of the monoterpenes.

CHAPTER IV

SEASONAL DEVELOPMENT OF MONOTERPENES IN FOLIAGE, STEMS, AND CONES OF BLUE SPRUCE

The different organs of blue spruce vary in their composition and concentrations of volatile oil. The development of monoterpene composition during the growing season in plant organs of differing composition may provide information on control of biosynthetic pathways. Since monoterpenes are closely related to other important compounds in the plant, an understanding of the control of monoterpene biosynthesis may also provide a better understanding of the synthesis of steroids, carotenoids, and other groups of isoprenoid compounds.

Seasonal development of the volatile oil of foliage has been studied in several species. Levinson et al. (1971) followed the changes that occurred in giant sequoia (Sequoiadendron giganteum (Lindl.) Decne.). They reported allyl phenyl ethers to be absent in new foliage, but they appeared as the growing season progressed. Maarse and Kepner (1970) reported an almost complete absence of cis-osimene, myrcene, and acyclic oxygenated monoterpenes in the new growth of Douglas-fir, which increased gradually in concentration over the growing season. In contrast, the cyclic oxygenated monoterpenes were immediately present in the new growth in amounts equivalent to those found in mature foliage. The monoterpene composition of the foliage of white spruce (Von Rudloff, 1972), sitka spruce (Hrutfiord et al., 1974), black spruce (Von Rudloff, 1975b), and blue spruce (Von Rudloff, 1975c) have

also been followed throughout the growing season.

The volatile oil of blue spruce foliage is high in camphene, limonene, and oxygenated monoterpenes. The stem oil is high in 3-carene, α-pinene, and β-pinene. The composition of the cone volatile oil of blue spruce is unreported. The cones exude resins during the growing season, in contrast to stem and foliage. The volatile oils of cones of sugar pine (Pinus lambertiana Dougl.) and ponderosa pine differed from the leaf and twig oils (Schroger, 1914). The similarities and differences in the patterns of development of the characteristic volatile oil composition of these organs may provide information on the control of the pathways that are involved, and changes in the levels of the volatile oils may be associated with developmental changes.

Trees of the same species may follow different developmental patterns. Most studies on seasonal development of the volatile oil composition have been on a single tree and a single tissue. In this study the composition of the volatile oil of three different organs of twelve mature blue spruce were studied.

Materials and Methods

Twelve mature blue spruce 10 meters in height on the Michigan State University campus were sampled at the mid-crown during the 1978 growing season. Vegetative buds were collected at bud break, May 19. Subsequent samples were separated into needles and stems. The developing branches were sampled ten times during the growing season until August 18. Only the current, developing growth was analyzed. Beginning June 5 the samples were dipped in liquid nitrogen to facilitate the removal of the needles. Prior to this time the needles could be

removed more easily from fresh, unfrozen samples. The samples were stored at -20°C until distillation.

Seven collections of cones were made from the same trees beginning with receptive strobili on May 22 through mature cones August 18. Several strobili were collected from each tree early in the growing season and single cones from each tree after June 7. Samples were stored at -20°C until distillation.

Duplicate samples were collected for all tissues at each collection time. The duplicate sample was oven dried at 70°C until a constant weight was obtained. The dry weight/fresh weight ratio was calculated. This factor was used to convert the yield of monoterpenes into µl/g dry weight of tissue.

Steam distillation was carried out as described in chapter 2. The needle and stem extracts were concentrated to 1 ml and the cone extracts concentrated to 5 ml.

Major monoterpenes were identified by gas chromatography-mass spectrometry on an LKB 9000 mass spectrometer. For routine work the peaks were identified by their retention times.

Results and Discussion

Patterns of development of needle volatile oil

Needle elongation occurred in a very brief period (figure 5). Bud break in 1978 for these trees was May 19 and the needles had reached their full extension by May 30. During the period of needle elongation there was little change in the total amounts of monoterpenes per gram of dry weight tissue (figure 5). When the concentration of monoterpenes was computed on a fresh weight basis there was a decrease in

Figure 5. Patterns of needle development for 12 blue spruce trees showing changes in mean needle length, mean concentration of total foliar monoterpenes expressed as µl/g dry weight (Δ) and µl/g fresh weight (□), and concentrations of bornyl acetate and 3-carene expressed as µl/g dry weight (Δ), µl/g fresh weight (□), and as a percentage of the total monoterpenes (*).

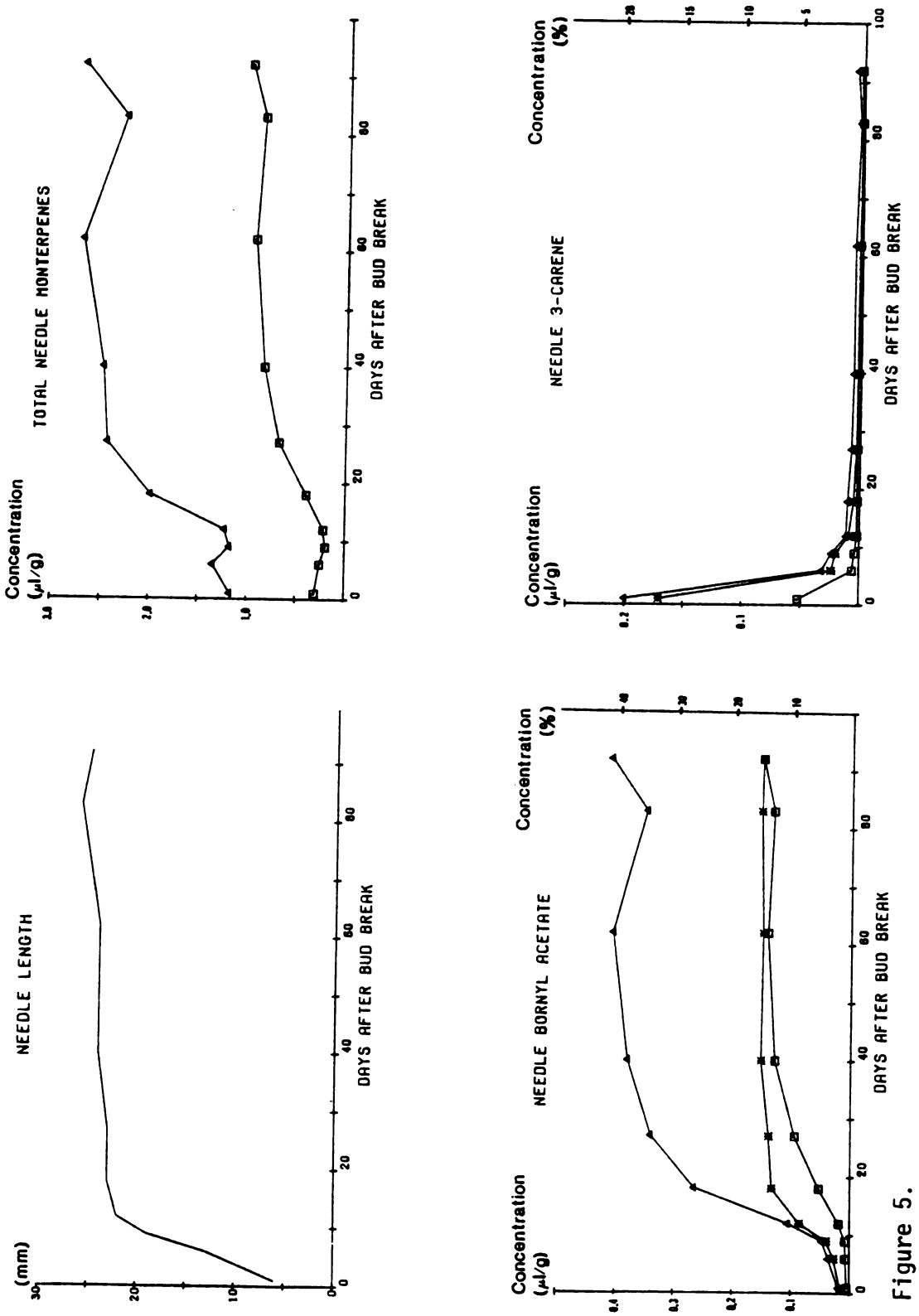

TOTAL NEEDLE MONTERPENES
Concentration (µl/g)
DAYS AFTER BUD BREAK
NEEDLE 3-CARENE
Concentration (%)
Concentration (µl/g)
DAYS AFTER BUD BREAK
NEEDLE LENGTH
(mm)
DAYS AFTER BUD BREAK
NEEDLE BORNYL ACETATE
Concentration (%)
Concentration (µl/g)
DAYS AFTER BUD BREAK

Figure 5.

the amounts of monoterpenes over the same period of time. The concentration of monoterpenes increased rapidly over the 15 days following needle elongation, with little change over the remainder of the growing season.

These results indicate that monoterpenes are present in very young needles. Owens (1968) reported the resin ducts were one of the first tissues to differentiate in Douglas-fir with the resin ducts reaching their full diameter before the needle is fully mature. In blue spruce the resin ducts of the needle appear to be functional in very young needles, and the foliage reaches the maximum monoterpene concentration early in the growing season. Zavarin et al. (1971a) reported that the monoterpene level characteristic of mature needles was reached before needle elongation was completed in ponderosa pine. In blue spruce the mature needle levels were reached only after needle elongation ceased.

The concentrations of the total oils changed over the growing season, obscuring changes in the composition of the oil. However, when the data was expressed on a percentage basis, it showed four seasonal patterns.

The first pattern, typified by the percentage of bornyl acetate (figure 5), was a rapid increase followed by constant levels through the remainder of the growing season. The other compounds that followed this pattern were tricyclene, camphene, β-phellandrene, terpinolene, and all of the oxygenated monoterpenes. With the exception of bornyl acetate, the oxygenated compounds were not present in detectable concentrations in the bud samples. The other monoterpenes of the first

group were present in the buds.

The order of appearance of the compounds may give support to hypothesized biosynthetic relationships. The final product of a biosynthetic pathway would appear only after the formation of an intermediate in the pathway. However, if the conversion of an intermediate to the final product is rapid and irreversible, the intermediate may not be detected. With samples collected days apart an entire biosynthetic group of compounds could appear for the first time at one collection time. Alpha-terpineol was the first of the oxygenated compounds, absent in the buds, to increase to detectable levels. Borneol and camphor were detected in the next collection, followed by terpinen-4-ol, and lastly detection of 1:8 cineole. This is in agreement with the proposed biosynthetic pathway given by Von Rudloff (1975a). In this scheme α-terpineol is formed from the same carbonium ion as limonene, and 1:8 cineole from α-terpineol. The order of detection of the compounds agrees with this biosynthetic order. Borneol and camphor, it is proposed, are formed from a different branch of the biosynthetic pathway with camphor being formed from borneol.

The second group of compounds, typified by 3-carene (figure 5), decreased rapidly after bud break. The other compound that followed this pattern was β-pinene. Higher percentages of both compounds were present in the stem than in the foliage. These compounds may be produced in large amounts by needles only in the bud. The rapid decrease in the concentrations of these compounds may be the result of conversion of these compounds, loss to the atmosphere, or dilution in the rapid formation of tissue and other monoterpenes.

The third pattern was typified by myrcene, which increased rapidly after bud break and then decreased throughout the remainder of the growing season (figure 6). Other compounds that followed similar patterns were santene, limonene, and several unknown compounds. The unknown compounds consisted of four chromatographic peaks of C_{15} hydrocarbons and alcohols that may correspond to cadinene and muurolene isomers and their alcohols reported to be present in blue spruce foliage by Von Rudloff (1975c). The rapid decline of the compounds of this group could be due to similar factors as affected the biosynthesis of 3-carene and β-pinene. The transient increase in the percentage of these compounds could be the result of their being intermediate compounds in a biosynthetic pathway. The intermediate compounds may be produced more rapidly than they can be converted to the next compound in the biosynthetic pathway early in the growing season. Another alternative could be the production of these compounds in young foliage and then a cessation, or drastic reduction, in production. This would be similar to 3-carene and β-pinene except that the third pattern compounds are produced in young needles instead of only in the buds. In contrast to the other compounds of this group limonene decreased gradually in percentage, but not in amounts on a dry weight basis.

The fourth pattern was shown only by α-pinene, a combination of the first and second patterns. The levels of α-pinene decreased rapidly and then increased and remained relatively constant over the remainder of the growing season. Similarly to 3-carene and β-pinene, α-pinene could be formed in the bud and not in the young needles, but in contrast to these compounds, as the needles mature a pathway for

Figure 6. Patterns of needle development for 12 blue spruce trees showing changes in the concentration of myrcene expressed as µl/g dry weight (△), µl/g fresh weight (□), and as a percentage of the total monoterpenes (*), and the seasonal variation for each of 12 trees for the concentration of total monoterpenes expressed as µl/g dry weight and for bornyl acetate expressed both as µl/g dry weight and as a percentage of the total monoterpenes.

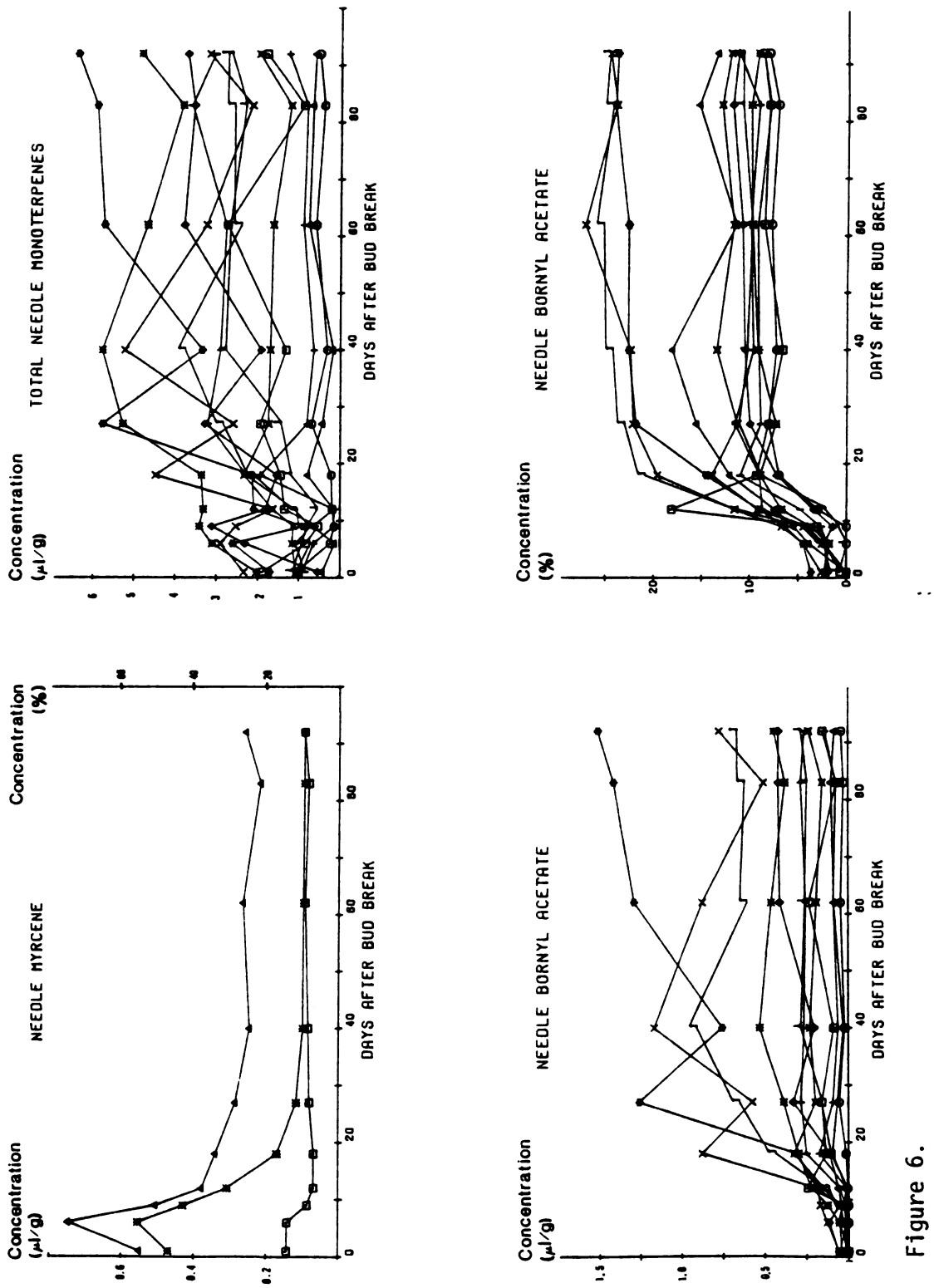

Concentration (µl/g)
TOTAL NEEDLE MONOTERPENES
DAYS AFTER BUD BREAK
Concentration (%)
NEEDLE BORNYL ACETATE
DAYS AFTER BUD BREAK
Concentration (µl/g)
NEEDLE MYRCENE
DAYS AFTER BUD BREAK
Concentration (µl/g)
NEEDLE BORNYL ACETATE
DAYS AFTER BUD BREAK

Figure 6.

the formation of α-pinene in the needles becomes operational.

The seasonal patterns of the percentages of the monoterpenes in the study agree with those reported by Von Rudloff (1975c) for a single tree. However, there were large differences among trees in the total concentrations of monoterpenes in the needles. The tree with the highest levels of total monoterpenes was 12 times that found in the tree with the lowest levels. Although the amounts of oil obtained from a given tree fluctuated from collection to collection, the differences among the trees in total concentration of monoterpenes became apparent shortly after bud break (figure 6). There were no distinct groupings of monoterpene yield, but a continuous variation in yield.

When the oil composition was expressed as the percentage of the total monoterpenes, rather than the yield of each individual monoterpene, the variation for some compounds did not appear to be continuous. There appeared to be distinct groupings at certain levels. It may be that the compounds have a continuous range of percentages, but appear to occur at specific levels because only 12 trees were sampled.

One possible reason for the groupings of the percentages at certain levels could be genetic control of the monoterpene level. Von Rudloff (1972) reported stable percentages of the composition of the volatile oil of white spruce to foliage over a five-year period, however, he reported some variation in total yield from year to year. Langenheim et al. (1979) reported high stability in both the yields and the percentage composition of the sesquiterpene hydrocarbons in Hymenaea courbaril. Langenheim et al. (1978) reported differences in the leaf blade resin pocket distribution and density among populations of Hymenaea courbaril. Therefore, both the amounts per gram of the

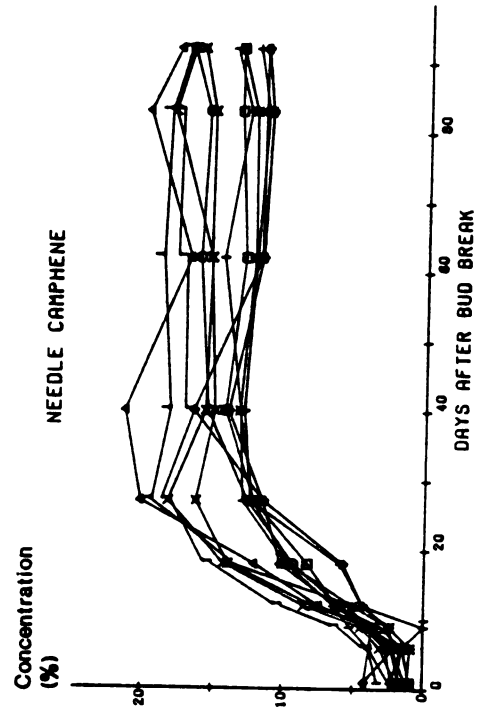
volatile oil in the leaves and the composition may be under genetic control, although the amounts per gram may be more subject to environmental variation. If both the amounts per gram and the composition of the oil are under genetic control, two systems of control of foliar monoterpenes would be involved. One system would control the fraction of the total oil that one monoterpene comprises and another system controls the amounts of oil produced per gram of tissue. The possible existence of these systems of genetic control should be explored in future studies.

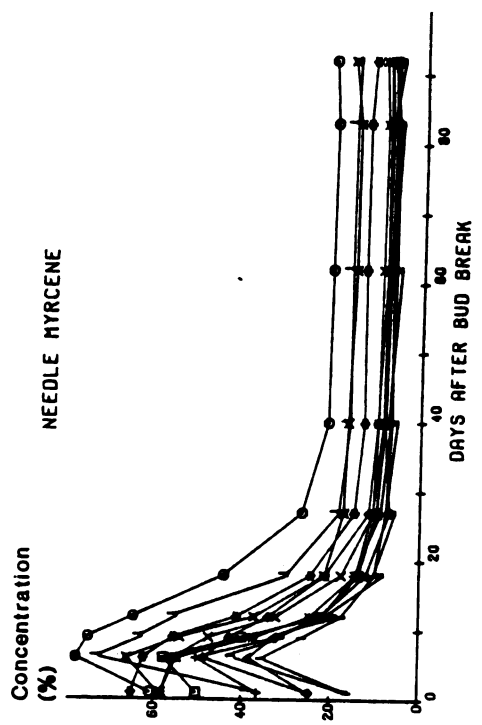
The wide range in amounts of total monoterpenes in the different trees obscures the groupings into distinct clusters that are apparent when expressed as a percentage of the total monoterpenes. This is illustrated by bornyl acetate (figure 6). When the concentrations of bornyl acetate are expressed as μl per gram of dry weight tissue there were no distinct groups. However, when bornyl acetate concentrations are expressed as a percentage of the total monoterpenes there are distinct groupings at 12 and 25 percent. Other compounds that suggest there may be different chemotypes are tricyclene, camphene, camphor, and myrcene (figure 7). The higher levels of these monoterpenes appeared to be due to a more rapid production rather than a longer time of production or a second cycle of synthesis later in the growing season.

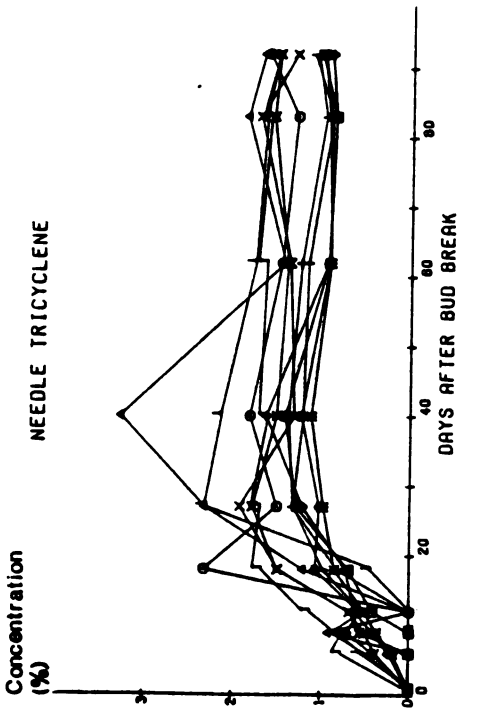
Patterns of development of stem volatile oil

Stem elongation occurred over a longer period of time than needle elongation. Stems reached full extension by June 14 in 1978, 26 days after bud break (figure 8). The amounts of total monoterpenes per gram of dry weight tissue increased rapidly in the eight days after bud break,

Figure 7. Patterns of needle development for 12 blue spruce trees showing seasonal variation in concentration of foliar monoterpenes for each of 12 trees for tricyclene, camphene, camphor, and myrcene expressed as a percentage of the total monoterpenes.


NEEDLE CAMPHENE
Concentration (%)
DAYS AFTER BUD BREAK


NEEDLE MYRCENE
Concentration (%)
DAYS AFTER BUD BREAK


NEEDLE TRICYCLENE
Concentration (%)
DAYS AFTER BUD BREAK

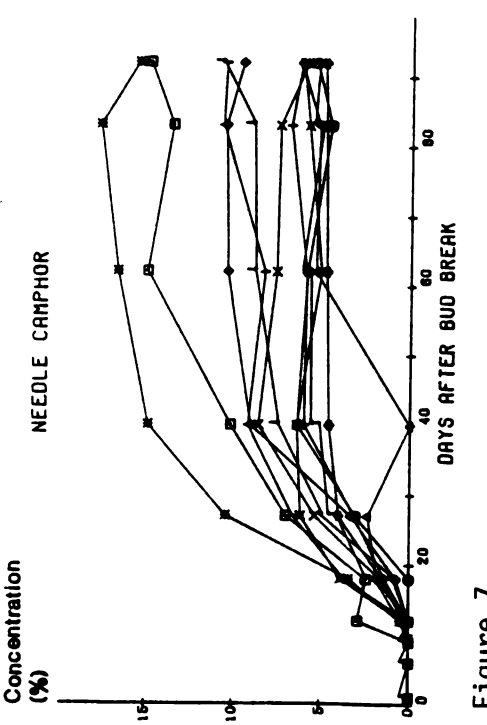

NEEDLE CAMPHOR
Concentration (%)
DAYS AFTER BUD BREAK

Figure 7.

Figure 8. Patterns of stem development for 12 blue spruce trees showing changes in the mean stem length, mean concentration of total stem monoterpenes expressed as µl/g dry weight (△) and µl/g fresh weight (□), seasonal variation in the concentrations of total stem monoterpenes for each of 12 trees expressed as µl/g dry weight, and mean concentrations of bornyl acetate expressed as µl/g dry weight (△), µl/g fresh weight (□), and as a percentage of the total monoterpenes (*).

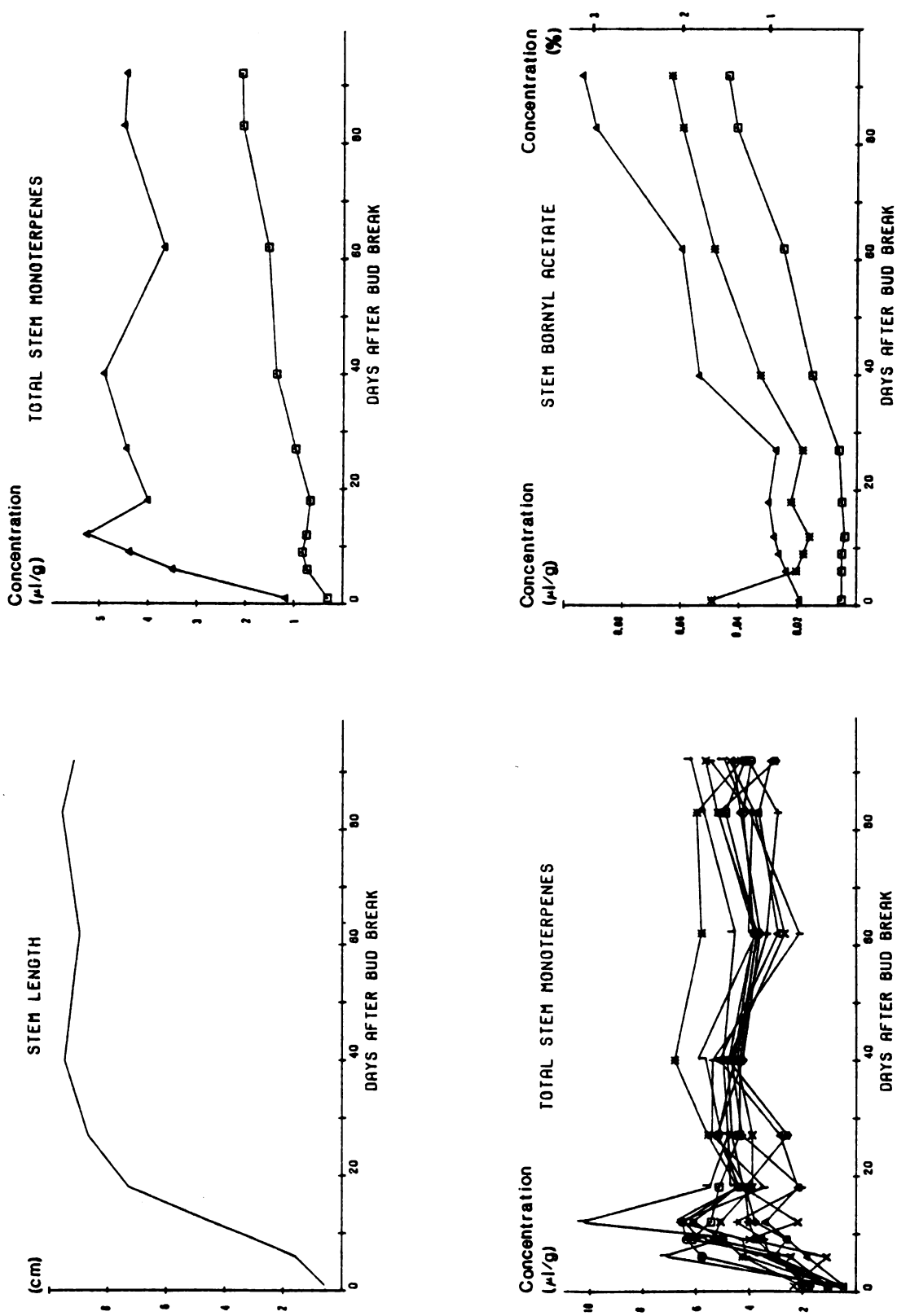

STEM LENGTH
(cm)
DAYS AFTER BUD BREAK
TOTAL STEM MONOTERPENES
Concentration
(µl/g)
DAYS AFTER BUD BREAK
STEM BORNYL ACETATE
Concentration
(%)
DAYS AFTER BUD BREAK
TOTAL STEM MONOTERPENES
Concentration
(µl/g)
DAYS AFTER BUD BREAK

Figure 8.

and then remained relatively constant (figure 8). On a fresh weight basis the amounts continued to increase throughout the growing season. Total amounts of monoterpenes in the stems varied less from tree to tree than did the total amounts in the needles (figure 8). As with the needles there were distinctive patterns of seasonal development when the oil composition was expressed as a percentage of the total monoterpenes.

The first pattern was typified by bornyl acetate, which decreased rapidly immediately after bud break and then gradually increased later in the growing season (figure 8). Myrcene followed a modification of this pattern, not showing an increase in percentage, but gradually decreased in percentage after stem elongation ceased. However, the amounts of myrcene on a dry weight basis increased rapidly in the first eight days after bud break and then decreased rapidly. Therefore, the decrease in the percentage from the first collection to the second was not due to a reduction in the concentration of myrcene, but a more rapid synthesis of other monoterpenes. Alpha-pinene, camphene, and β-pinene all follow very similar patterns, decreasing rapidly in percentage after bud break with an increase after stem elongation ceases. As with myrcene, the actual concentrations of these compounds increased while the percentage was rapidly decreasing.

The second pattern was typified by 3-carene and α-terpineol (figure 9). These compounds increased early in the growing season and then decreased in percentage through the rest of the growing season, similar to limonene and myrcene in the needles. There were two groups of stem monoterpenes that followed the second pattern. The percentages of 3-carene, sabinene, limonene, and terpinolene increased rapidly after

Figure 9. Patterns of stem development for 12 blue spruce trees showing changes in the mean concentrations of 3-carene, α-terpineol, and β-phellandrene expressed as µl/g dry weight (△), µl/g fresh weight (□), and as a percentage of the total monoterpenes (*), and unknown compound 25 expressed as µl/g dry weight (△) and µl/g fresh weight (□).

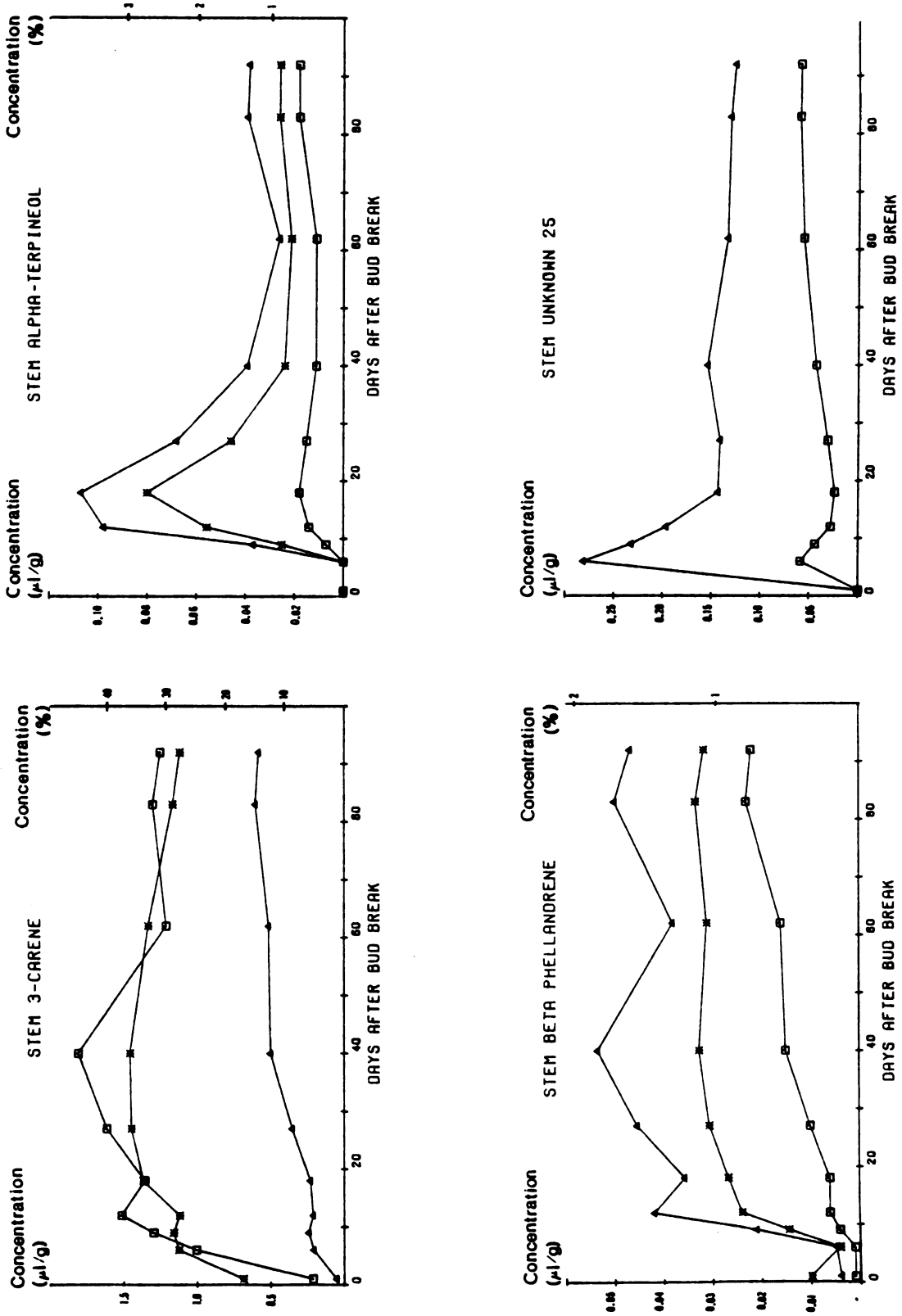

STEM ALPHA-TERPINEOL
Concentration (µl/g)
Concentration (%)
DAYS AFTER BUD BREAK
STEM UNKNOWN 25
Concentration (µl/g)
DAYS AFTER BUD BREAK
STEM 3-CARENE
Concentration (µl/g)
Concentration (%)
DAYS AFTER BUD BREAK
STEM BETA PHELLANDRENE
Concentration (µl/g)
Concentration (%)
DAYS AFTER BUD BREAK

Figure 9.

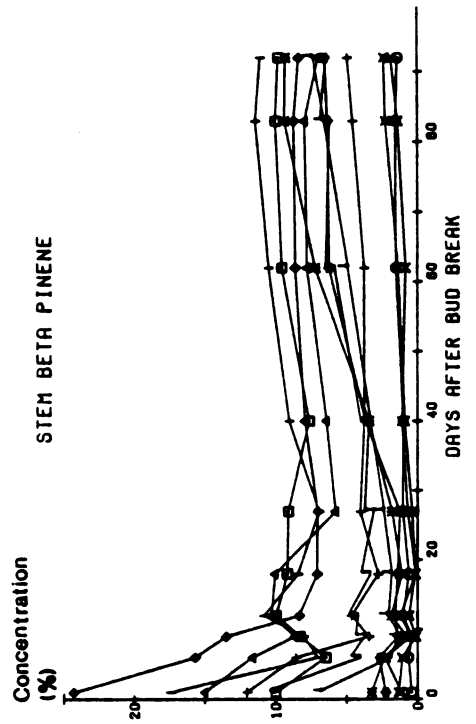
bud break and then declined slowly. The percentages of α-terpineol and terpinen-4-ol decreased more rapidly.

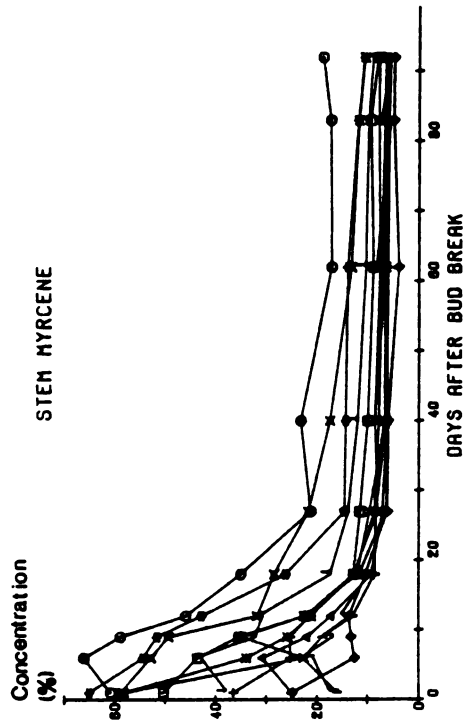
The third pattern was typified by β-phellandrene (figure 9). Beta-phellandrene and γ-terpinene increased in percentage rapidly and then reached a constant level. This pattern was similar to that of bornyl acetate in the needles. This pattern is consistent with compounds that are produced in minimal amounts in the bud and larger amounts in the stem.

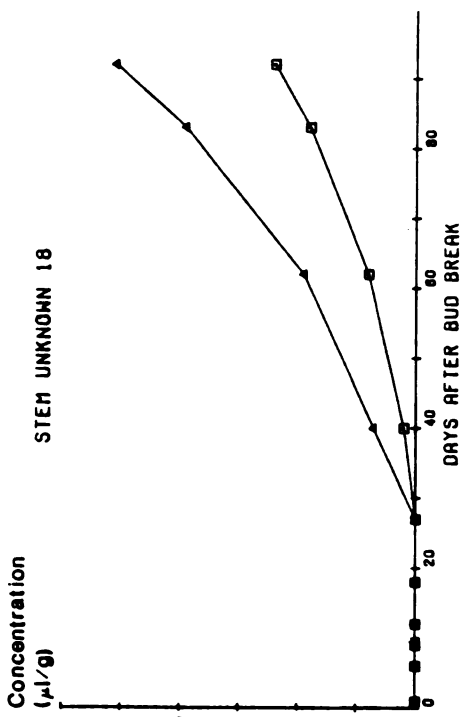
There were large concentrations of unknown high molecular weight volatile compounds that were not present in appreciable concentrations in the needles. These unknown compounds were not reported by Von Rudloff (1975c) in his analysis of blue spruce twigs. These unknowns followed two major patterns that intergraded. The first pattern was a rapid increase in concentration early in the growing season and then decreased (figure 9). The other pattern consisted of compounds that became major components of the volatile oil only late in the growing season (figure 10). Some unknown compounds combined the two patterns and increased rapidly, then decreased or remained constant, and increased at the end of the growing season.

As in needles, there were groupings of the percentages for some of the monoterpene levels. One tree had a higher percentage of myrcene and sabinene than any of the other trees (figure 10). The seasonal trends for the percentage of myrcene and sabinene were similar for all 12 trees. The tree with the highest levels of myrcene in the stem also had the highest percentage of myrcene in the needles. There also appeared to be different β-pinene chemotypes (figure 10). At the end

Figure 10. Patterns of stem development for 12 blue spruce trees showing changes in the mean concentration of unknown expressed as µl/g dry weight (△) and as µl/g fresh weight (□), and seasonal variation in concentration of stem monoterpenes for myrcene, sabinene, and β-pinene for each of 12 trees expressed as a percentage of the total monoterpenes.


STEM BETA PINENE
Concentration (%)
DAYS AFTER BUD BREAK


STEM MYRCENE
Concentration (%)
DAYS AFTER BUD BREAK


STEM UNKNOWN 18
Concentration (µl/g)
DAYS AFTER BUD BREAK

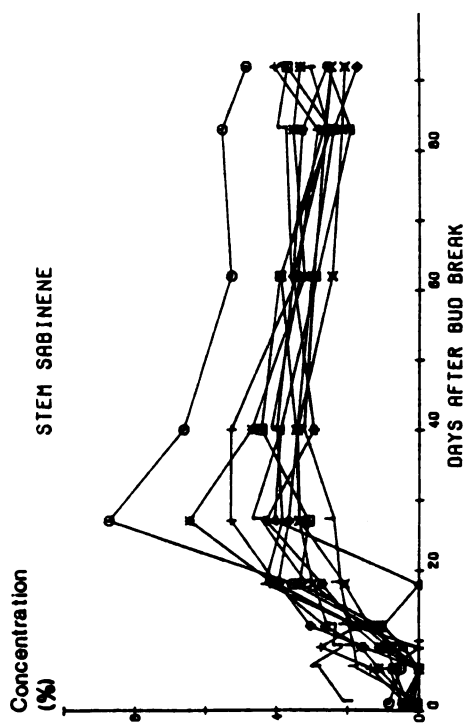

STEM SABINENE
Concentration (%)
DAYS AFTER BUD BREAK

Figure 10.

of the growing season there were three trees that had low levels of β-pinene and nine that had high levels. Five of the trees that had high levels at the end of the growing season decreased in percentage immediately after bud break, but remained at a higher level of β-pinene than the other trees. The other four trees that had high levels of β-pinene at the end of the growing season, decreased in percentage of β-pinene to low levels after bud break and after cessation of stem elongation increased in percentage. In contrast to the other monoterpenes with possible chemotypes, the levels of β-pinene in the stem may be due to mechanisms acting at different times rather than different efficiencies of production of β-pinene.

Patterns of development of cone volatile oil

The fresh weight of the cone increased linearly for 40 days after vegetative bud break, and then was constant (figure 11). Initially the strobili were upright on the branches, but later in the growing season they became pendant. Collections of the cones corresponded with developmental changes. Collections were made when the strobili were receptive, at scale closure, and when the cones became pendant. The fourth collection was at the time when fertilization takes place (Hanover, 1975b). The seeds were fully developed and germinated normally by the fifth collection, although the cones would not open upon drying. The cones began to turn brown and would open normally at the time of the sixth collection and were brown by the last collection.

The seasonal development of monoterpenes followed a different pattern in cones than either needles or stems (figure 11). The among-tree variation was similar to that found for stem volatile oils

Figure 11. Patterns of cone development for 12 blue spruce trees showing changes in the mean cone fresh weight, mean concentrations of total cone monoterpenes expressed as µl/g dry weight (△) and µl/g fresh weight (□), and seasonal variation in the concentrations of total cone monoterpenes for each of 12 trees expressed both as µl/g fresh weight and µl/g dry weight.

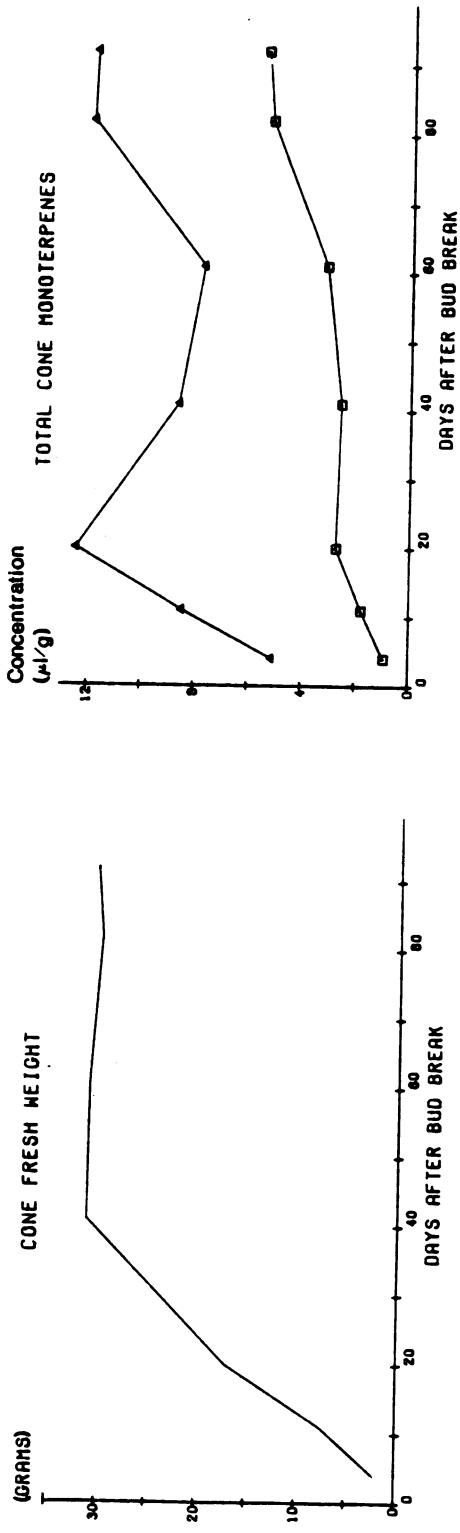
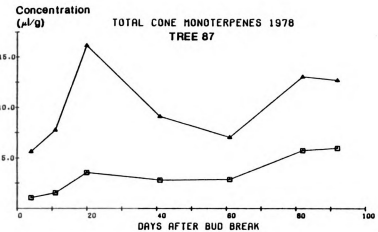

CONE FRESH WEIGHT
(GRAMS)
DAYS AFTER BUD BREAK
TOTAL CONE MONOTERPENES
Concentration (µl/g)
DAYS AFTER BUD BREAK
TOTAL CONE MONOTERPENES
Concentration (µl/g DW)
DAYS AFTER BUD BREAK
TOTAL CONE MONOTERPENES
Concentration (µl/g FW)
DAYS AFTER BUD BREAK

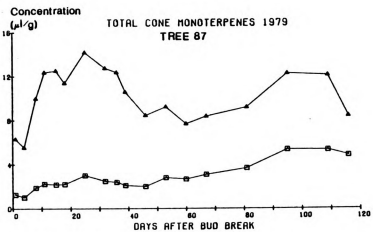
Figure 11.

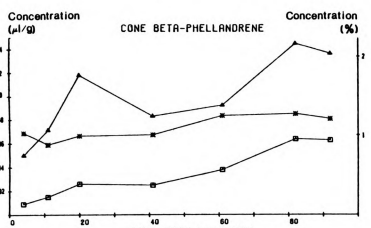
(figure 11). The amounts of monoterpenes increased rapidly when computed on a dry weight basis, reaching a peak when the cones became pendant then decreasing until the seeds were capable of germinating, at which time the amounts increased again. Seeds were steam distilled and only small amounts of monoterpenes were found, so the second increase in the concentration of monoterpenes was not due to synthesis of monoterpenes in the seeds. When the concentrations were determined on a fresh weight basis the first peak in concentration was not as pronounced. This was due to differences in the time when the first maximum occurred in the trees, although all trees followed the same pattern (figure 11). Because the dry weight to fresh weight ratio is lower early in the growing season the first maximum is more apparent on a dry weight basis. To verify that the pattern was typical of cone development, collections of cones were made from one tree in the 1979 growing season. Cones from this tree showed the same pattern for all compounds and total monoterpenes in both 1978 and 1979 (figure 12). The concentrations of total monoterpenes were similar in both years even though the strobili were receptive 12 days earlier in 1979, and the cones were 65% of the fresh weight of the 1978 cones from the same tree.

The concentrations of most of the compounds followed the same pattern as the total monoterpenes. However, the maxima were not as pronounced for some compounds as they were for the total monoterpenes. Myrcene and 3-carene decreased in amount per gram dry weight and showed a very slight increase in concentration at the end of the growing season. When the oil composition was expressed as a percentage of the total monoterpenes, different patterns of development were evident.

Figure 12. Patterns of cone development for 12 blue spruce trees showing the seasonal pattern for concentrations of total cone monoterpenes expressed as µl/g dry weight (△) and µl/g fresh weight (□) for tree 87 in 1978 and 1979, and mean concentrations of β-phellandrene the myrcene expressed as µl/g dry weight (△), µl/g fresh weight (□), and as a percentage of the total monoterpenes (*).


Concentration
(µl/g)
TOTAL CONE MONOTERPENES 1978
TREE 87
15.0
10.0
5.0
0
20
40
60
80
100
DAYS AFTER BUD BREAK


Concentration
(µl/g)
TOTAL CONE MONOTERPENES 1979
TREE 87
12
8
4
0
20
40
60
80
100
120
DAYS AFTER BUD BREAK


Concentration
(µl/g)
CONE BETA-PHELLANDRENE
Concentration
(%)
0.14
0.12
0.10
0.08
0.06
0.04
0.02
2
1
0
20
40
60
80
DAYS AFTER BUD BREAK

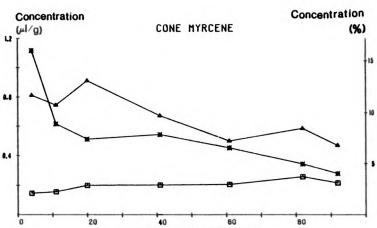

Concentration
(µl/g)
CONE MYRCENE
Concentration
(%)
1.2
0.8
0.4
15
10
5
0
20
40
60
80
DAYS AFTER BUD BREAK

Figure 12.

The first pattern was typified by β-phellandrene (figure 12). Both β-phellandrene and camphene showed little seasonal variation in percentage over the growing season.

The second pattern, typified by myrcene (figure 12), was similar to that shown by needle 3-carene and β-pinene. The other compounds that followed this pattern were 3-carene and terpinolene. The 1979 collections were made at earlier stages of development than those of 1978, and in the early samples myrcene was present in cones in the highest proportion.

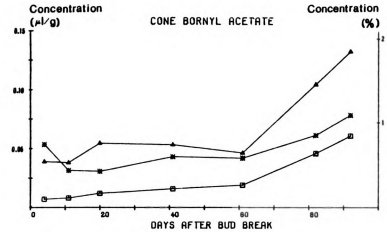
The third pattern was typified by bornyl acetate, (figure 13) which was similar to the pattern of α-pinene in the stem. The percentage of these compounds decreased early in the growing season and increased as the cones matured. Other compounds of this group were γ-terpinene and the oxygenated compounds terpinen-4-ol and α-terpineol.

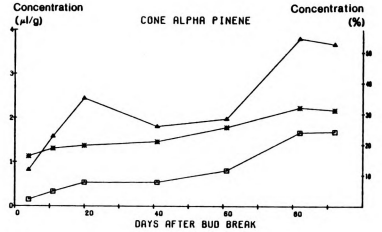
The fourth pattern was typified by α-pinene (figure 13). Both α-and β-pinene showed a continual increase in percentage.

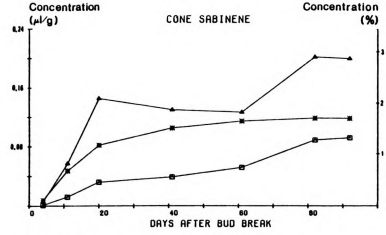
Sabinene typified the fifth pattern (figure 13), which was similar to that shown by needle bornyl acetate and stem β-phellandrene. Sabinene increased in percentage and then reached a stable level. Limonene was similar, but showed a slight gradual decline in percentage.

Large concentrations of unknown high molecular weight volatile compounds were found. The major cone unknown compounds had longer retention times on a SE-30 column than the major stem unknown compounds. Preliminary attempts to identify these compounds in the stem and cone by examination of their mass spectra were not successful. Three basic

Figure 13. Patterns of cone development for 12 blue spruce trees showing changes in the mean concentration of bornyl acetate, α-pinene, sabinene expressed as µl/g dry weight (△), µl/g fresh weight (□), and as a percentage of the total monoterpenes (△), and unknown compound 25 expressed as µl/g dry weight (□) and µl/g fresh weight (*).


Concentration
(µl/g)
CONE BORNYL ACETATE
Concentration
(%)
0.15
0.10
0.05
2
1
0
20
40
60
80
DAYS AFTER BUD BREAK


Concentration
(µl/g)
CONE ALPHA PINENE
Concentration
(%)
4
3
2
1
0
40
30
20
10
0
20
40
60
80
DAYS AFTER BUD BREAK


Concentration
(µl/g)
CONE SABINENE
Concentration
(%)
0.24
0.16
0.08
3
2
1
0
20
40
60
80
DAYS AFTER BUD BREAK

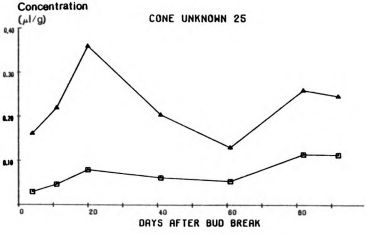

Concentration
(µl/g)
CONE UNKNOWN 25
0.40
0.30
0.20
0.10
0
20
40
60
80
DAYS AFTER BUD BREAK

Figure 13.

patterns are shown by the cone unknowns in the amounts of volatile per gram of dry weight tissue. Some of the unknowns showed the same pattern as the total monoterpenes (figure 13). The second group showed little change in concentration in the early part of the growing season, but increased rapidly at the end of the growing season (figure 14). Unknown compound 23 reached a single maximum in the middle of the growing season then decreased to the original levels at the end of the growing season (figure 14).

It is difficult to postulate mechanisms for the irregular patterns of the amounts of the cone monoterpenes per gram of tissue over the growing season. Changes in the levels of the monoterpenes can be better understood when the amounts of monoterpenes in the entire cone are calculated. The production of total monoterpenes per cone was almost linear over the growing season (figure 14). There was a slight decrease in the slope 20 to 60 days after bud break, but because the cone continues to grow, the amounts of monoterpenes per gram of tissue decreases. However, there does not appear to be a net loss of monoterpenes in the cone even though the amounts per gram decrease. Thus, the irregular pattern of concentration of total monoterpenes does not appear to be related to specific developmental changes, but rather to slight changes in the rates of production of monoterpenes and changes in the growth rates of the cones.

If monoterpenes are produced in cones at a constant rate per gram of tissue, there would be an increasing rate of accumulation of monoterpenes since the cone increased in size over most of the growing season. This was the pattern shown by camphor (figure 14), γ-terpinene,

Figure 14. Patterns of cone development for 12 blue spruce trees showing changes in mean concentration for unknown compounds 18 and 23 expressed as µl/g dry weight (△) and µl/g fresh weight (□), and mean concentration of total cone monoterpenes and camphor expressed as µl/cone.

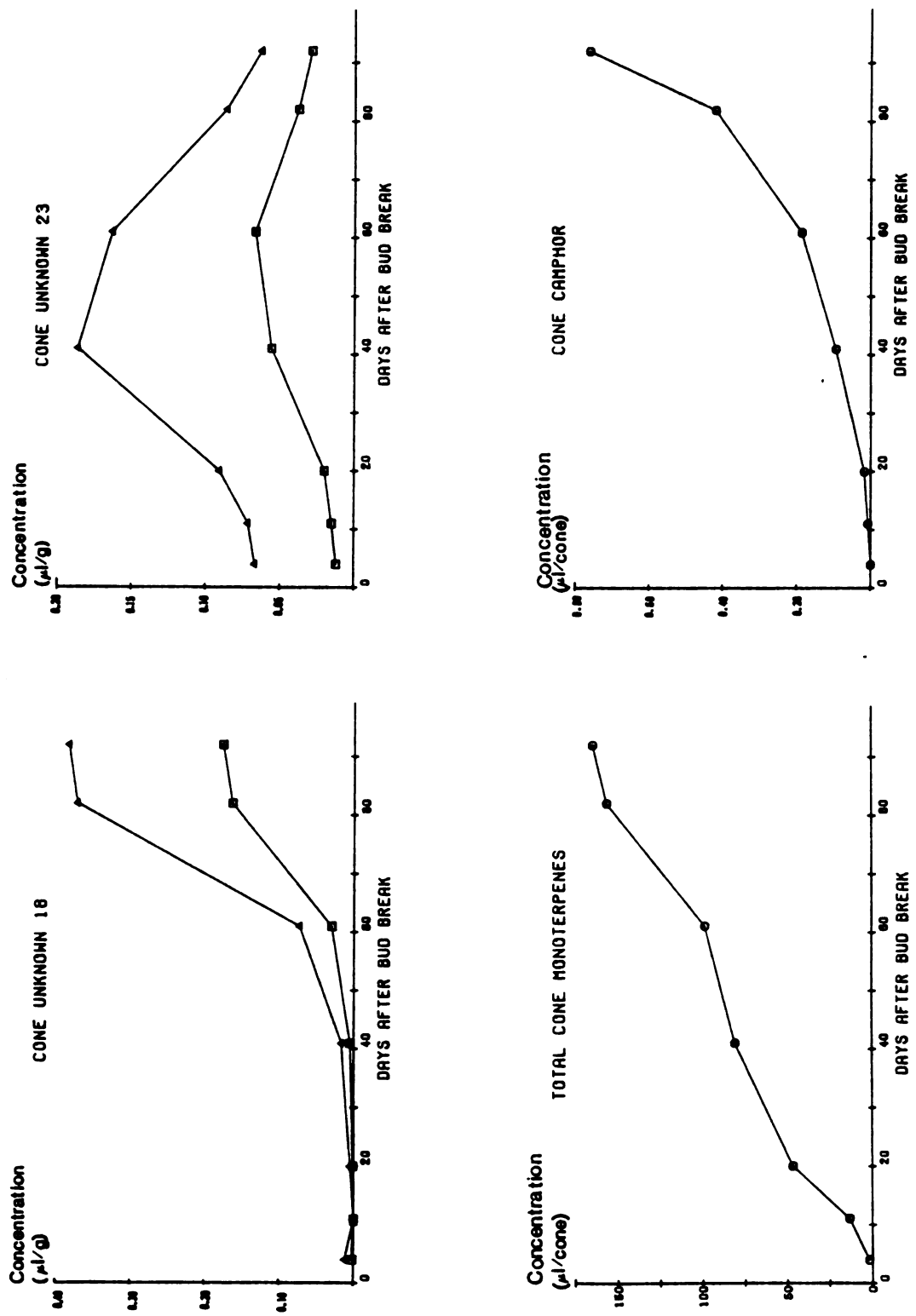

CONE UNKNOWN 23
Concentration (µl/g)
DAYS AFTER BUD BREAK
CONE CAMPHOR
Concentration (µl/cone)
DAYS AFTER BUD BREAK
CONE UNKNOWN 18
Concentration (µl/g)
DAYS AFTER BUD BREAK
TOTAL CONE MONOTERPENES
Concentration (µl/cone)
DAYS AFTER BUD BREAK

Figure 14.

terpinen-4-ol, α-terpineol, and unknown 18.

The second pattern of accumulation was typified by β-pinene (figure 15), which showed a linear increase in the amounts per cone. Other compounds that followed this pattern were α-pinene, camphene, sabinene, limonene, β-phellandrene, and unknowns 26 and 28. The increases shown by these compounds could not be explained by a constant production per unit of tissue, unless the producing area remained constant in size. A similar production curve would be produced if changing portions of the cone produced the compounds at a constant rate, or if the producing cells are regulated so as to decrease the rate of production as the cone becomes larger.

The third pattern was typified by unknown 24 (figure 15). This pattern was exhibited by bornyl acetate and unknown compounds 20, 21, and 22. These compounds accumulated at one rate through most of the growing season and a much higher rate later in the growing season. This suggests two different mechanisms of formation. One biosynthetic pathway might be operational early in the growing season and the second as the cone matures. Both would be regulated as the previous group of compounds were. Terpinolene and unknown 25 followed a similar pattern, except there was a period between the two rates of accumulation when there was no increase in amounts in the cone.

Myrcene typified the fourth pattern of accumulation (figure 15). Both myrcene and 3-carene increased in amounts early in the growing season and then did not increase over the remainder of the growing season. It would appear that myrcene was synthesized in the first 40 days after bud break and 3-carene in the first 20 days. After this time,

Figure 15. Patterns of cone development for 12 blue spruce trees showing changes in concentrations of β-pinene, unknown compound 24, myrcene, and unknown compound 23 expressed as µl/cone.

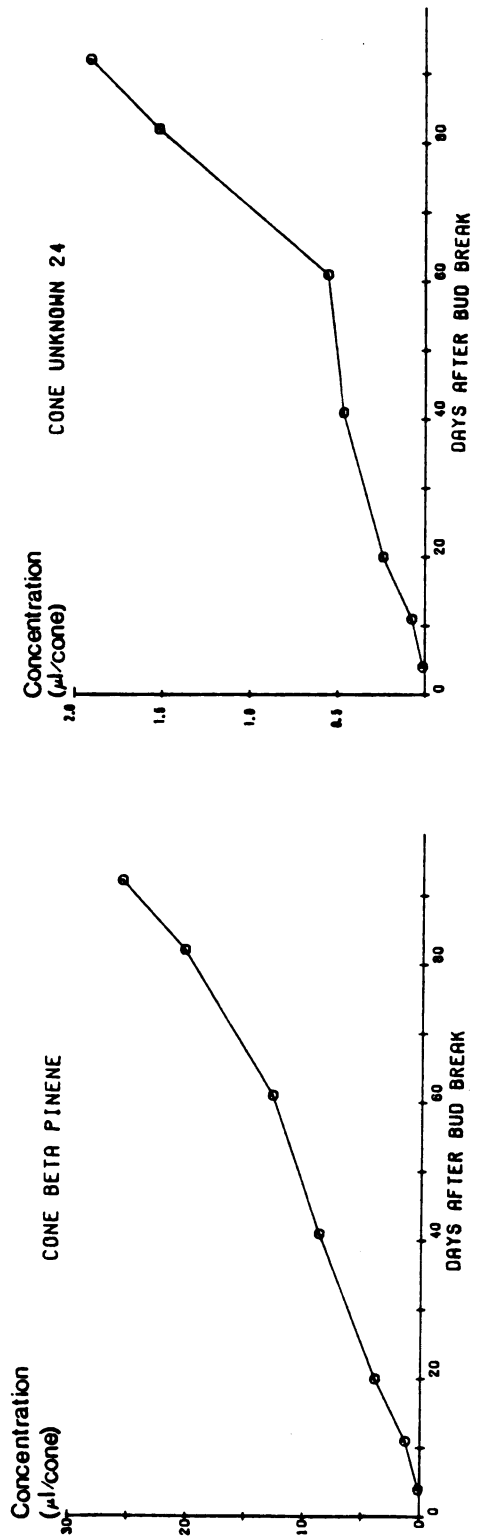

CONE BETA PINENE
Concentration (µl/cone)
DAYS AFTER BUD BREAK
CONE UNKNOWN 24
Concentration (µl/cone)
DAYS AFTER BUD BREAK
CONE MYRCENE
Concentration (µl/cone)
DAYS AFTER BUD BREAK
CONE UNKNOWN 23
Concentration (µl/cone)
DAYS AFTER BUD BREAK

Figure 15.

these pathways did not appear to result in the net production of these compounds. As a consequence these compounds decreased rapidly both as a percentage of the total monoterpenes and in amounts per gram of tissue.

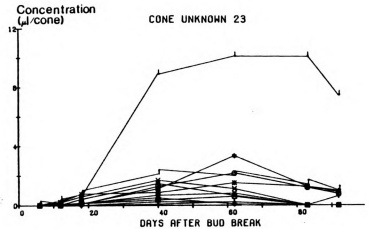
The pattern shown by unknown compound 23 was different from all of the other compounds (figure 15). The amounts of this compound in a cone reached a peak 60 days after bud break and then decreased as the cone matured. This suggests a loss or conversion. However, only one tree accumulated appreciable amounts of this compound (figure 16). This tree accumulated the unknown compound rapidly between 17 and 40 days after bud break and then remained relatively constant over the remainder of the growing season. The other 11 trees had low levels of the compound and the slight decrease observed in these trees could be due to experimental error.

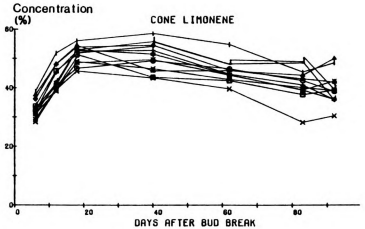
The changes in the concentration of compounds appeared to be due to altering the rate of production of the compound while the cone was increasing in weight rather than a loss or conversion of the compound. This does not eliminate the possibility of losses of these compounds or conversions to other compounds. However, there are no net losses of these compounds with the possible exception of unknown 23.

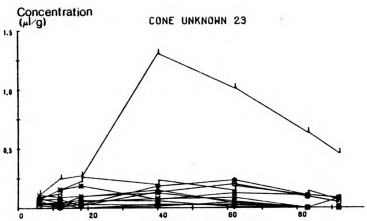
The same principles could be used with needles to determine if the rapid decreases in the levels of myrcene, β-pinene, and 3-carene are due to losses or conversions of these compounds or are the result of changes in the rates of formation of these compounds and dilution through growth of the needles.

Several compounds showed groupings at discrete levels. One tree had a lower percentage of limonene than any of the other 11 trees

Figure 16. Patterns of cone development for 12 blue spruce trees showing seasonal variation in concentration of cone volatile oil for each of 12 trees for unknown 23 expressed as µl/cone and µl/g dry weight, and limonene and β-pinene expressed as a percentage of the total monoterpenes.


Concentration
(µl/cone)
CONE UNKNOWN 23
12
8
4
0
0
20
40
60
80
DAYS AFTER BUD BREAK


Concentration
(%)
CONE LIMONENE
60
40
20
0
0
20
40
60
80
DAYS AFTER BUD BREAK


Concentration
(µl/g)
CONE UNKNOWN 23
1.5
1.0
0.5
0
0
20
40
60
80
DAYS AFTER BUD BREAK

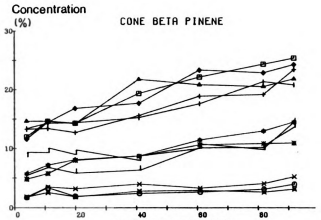

Concentration
(%)
CONE BETA PINENE
30
20
10
0
0
20
40
60
80
DAYS AFTER BUD BREAK

Figure 16.

(figure 16). One tree had a much higher concentration of unknown 23 than any other tree (figure 16). This tree started the growing season with slightly higher levels of unknown 23 in the cones and increased in amounts of this compound between 17 and 40 days after bud break. The other trees did not show a corresponding increase. The levels of cone β-pinene showed three distinct groupings at 4, 13 and 23% (figure 16). The trees in these groupings are the same as the three groupings of β-pinene in the stem. Those trees that were in the low group in the stem also composed the low group in the cones. The high group in the stem was composed of two subgroups. The first subgroup decreased in percentage and then increased midway through the growing season. The second subgroup decreased in percentage of β-pinene to a stable level that was the level that was later attained by the first subgroup. The first subgroup was composed of trees that had a cone β-pinene level of 13%. The second subgroup was composed of trees with a cone β-pinene level of 23%.

Comparison among organs

The seasonal patterns of total monoterpene concentration varied according to the organ studied. The composition of the volatile oil in needles, stems, and cones at the end of the growing season is given in table 19. The concentrations of total needle monoterpenes did not change on a dry weight basis during needle elongation. The concentrations then increased rapidly for a short period of time until reaching a stable level. The composition of the needle oil changed very rapidly over the same time period until a stable concentration of monoterpenes was reached; then there was little change in composition. Stem

Table 19.--Mean volatile oil composition of twelve blue spruce trees in different organs at end of growing season

Monoterpene	Tissue		
	Needle	Stem	Cone
	---Percent of total monoterpenes---		
Santene	.2	--	--
Tricyclene	1.1	--	--
α-pinene	8.2	23.3	31.1
Camphene	13.9	.8	.6
β-pinene	.7	6.3	15.7
Sabinene	.1	3.0	1.7
Myrcene	9.6	8.2	4.0
3-carene	.2	27.9	1.3
Limonene	30.3	17.6	40.0
β-phellandrene	.5	1.1	1.2
γ-terpinene	--	1.0	.3
1:8 cineole	.7	--	--
Terpinolene	1.0	4.9	.8
Camphor	8.9	.9	.5
Borneol	2.1	--	--
Terpinen-4-ol	.7	1.0	.6
α-terpineol	2.2	.9	1.0
Bornyl acetate	15.3	2.1	1.1
	--------μl/g dry weight---------		
Total unknown volatile compounds	.09	1.28	3.85
Total monoterpenes	2.70	4.48	11.81

monoterpenes rapidly increased in concentration immediately after bud break while the stems were elongating. The stems reached stable concentrations of total monoterpenes on a dry weight basis before the stem had fully elongated. Composition of stem oil changed after the stem elongation had been completed even though there were no changes in the total concentrations of monoterpenes. In contrast, concentrations of total monoterpenes in the cones did not reach a stable level. There was a maximum early in the growing season and concentrations were increasing at the end of the growing season. The cones were not a closed system but exuded resin. This would allow continuing synthesis and accumulation of large amounts of resin with no restrictions of volume. There did not appear to be a stable stage for sampling cone monoterpenes, expressed either as amounts per gram of tissue or percent of total monoterpenes. Therefore, cones would not be well suited for most genetic and chemosystematic studies.

Some monoterpenes showed similar developmental patterns in different organs when the monoterpene level was expressed as a percentage of the total monoterpenes. The pattern for myrcene and limonene was similar in all three organs sampled. In the needles, the percentage of myrcene increased sharply and then showed a rapid decline as in stems and cones. The high levels of myrcene in the young tissue may be a characteristic of spruce. Von Rudloff reported a sharp peak in the percentage of myrcene in the needles of blue spruce (1975c), white spruce (1972), and black spruce (1975a), and Hrutfiord et al. (1974) reported the same pattern in sitka spruce needles. It appears that the decrease in the percentage of myrcene in stems and

cones in this study was due to the increased production of monoterpenes relative to myrcene. In the cone, the decrease in the amounts of myrcene per gram of tissue was due to the cessation of production of myrcene and the continued growth of the cones.

There were some differences among organs in the pattern that a specific monoterpene followed. The pattern for bornyl acetate and α-terpineol were similar in the stem and cone, where they were not major components of the volatile oil. The pattern for these compounds was different in the foliage where they reached a higher proportion of the total oil. Beta-pinene increased in percentage in both stem and cones after the beginning of the growing season, while in needles the percentage decreased to very low levels. The pattern of 3-carene was similar in needles and in the cone, where it decreased in percentage as the organs developed. In the stem it increased in percentage to one of the major components. Alpha-pinene was a major component in all organs sampled, but followed a different developmental pattern in each. The percentage in the stem decreased at the beginning of the growing season and then followed the same pattern as in the cone. The pattern of α-pinene in the needle was different than in either the stem or the cone.

Conclusions

The developmental patterns of the individual monoterpenes varied among monoterpenes and organs, both in the concentrations and in their percentages. Some monoterpenes developed in the same pattern in all organs studied, as did myrcene and limonene. Others followed different patterns in all organs sampled, as did α-pinene. Concentrations of

total monoterpenes reached stable levels in the stem and needles on a dry weight basis, but not on a fresh weight basis. Concentrations of total monoterpenes in the cone did not reach a stable level at any time over the growing season. This may reflect the fact that the resin ducts of the needles and the stem represent a closed system, while the cone exudes resin and thus does not have a fixed volume.

The seasonal pattern for amounts of monoterpenes per gram of tissue in the cones was irregular, with two maxima and no stable period for sampling. However, when the total amounts of monoterpenes in a cone were determined, the increase over the growing season was essentially linear. Myrcene and 3-carene showed rapid decreases in the concentrations in the cone. However, when the amounts were calculated in the cone, there did not appear to be a loss of these compounds. The decrease appeared to be due to a decreased production of these monoterpenes while the cone continued growth and production of other monoterpenes. This method of calculating the amounts of a monoterpene in the biosynthetic unit may be useful in studying needle monoterpenes.

There were discrete groupings of the percentages of some of the monoterpenes in some tissues. These chemotypes may be due to genetic differences and could be of future interest. Levels of β-pinene differed greatly in the three organs studied. In the cones there appeared to be three distinct groupings of percentages of β-pinene. In the stem there were two distinct groupings of β-pinene levels. The group with a high percentage of β-pinene in the stem was composed of the two high percentage groups of the cones. In the needles the β-pinene level decreased rapidly in all trees. If these levels are controlled by the

same gene(s), further study might lead to better understanding of differential gene action in different tissues.

CHAPTER V

INTERRELATIONSHIPS AMONG MONOTERPENES

In the previous chapter, seasonal variation patterns of monoterpenes in developing organs were described. Several monoterpenes followed similar patterns and may be closely related biosynthetically. In this chapter the relationships among monoterpenes are studied by simple correlation analysis.

The use of correlations among monoterpenes to determine biosynthetic relationships has been discussed by Zavarin (1970) and Westfall (1972). An exact biological interpretation of correlations between two compounds is not possible. Many factors could create a relationship between two compounds. Even in a simple branched biosynthetic pathway, a range of possible correlations among compounds is possible. When compound A is converted to compound B which may be converted to two compounds C and D, there can be positive, negative, or no correlation between C and D. When the conversion of A to B is the controlling step in the synthesis of C and D, C and D would probably be positively correlated. If the controlling step were the conversion of B to C and D, correlations between C and D would depend on the relative efficiencies of the conversion of B and could range from near zero to a negative correlation. When the compounds are a part of a sequential conversion of one compound to another, as with A and B, the correlations could range from negative to positive, depending on the types of enzyme action.

When correlations are calculated between two compounds over the growing season the presence of a significant correlation could indicate the presence of a biosynthetic association between two compounds. Further determination of the biosynthetic relationship between the compounds cannot be determined solely from correlations. Changes in the correlation between two monoterpenes over the growing season could indicate changes in their relationship. This change could be the result of an alteration in the biosynthetic pathway of one or both of the compounds. These alterations could be the result of a gene being turned off or on during development. The change in the correlation between the compounds could result from changes in the existing biosynthetic pathways, or by an additional pathway becoming more active and causing a higher proportion of a compound to be formed by this pathway.

Materials and Methods

Needle, stem and cone samples were collected from 12 blue spruce trees on the Michigan State University campus and analyzed for monoterpenes as described in the previous study. Simple correlations were calculated between each pair of major monoterpenes within an organ at each collection time. Correlations were also calculated between the levels of the same monoterpene in different organs at each collection time. Correlations were calculated with monoterpene levels expressed both as µl/g dry weight and as a percentage of the total monoterpenes. The correlations of the percentages were computed with transformed data, using the arcsine transformation.

Results and Discussion

Twelve trees were sampled throughout the growing season. Because of the small numbers of trees sampled and the large number of correlations computed, some of the significant correlations could be due to sampling and not of biological significance. Because the percentage of the total monoterpenes must total 100% there is a correlation due to constraint, which can be calculated (Mosimann, 1962). The expected correlations due to constraint were calculated for correlations between compounds that comprised a large proportion of the total monoterpenes and deviations from this value tested. Correlations among some pairs of compounds at some individual collection times did not deviate significantly from those expected from correlations due to constraint. However, for all pairs of compounds that were significantly correlated for several collection times, correlations at some of the collection times deviated significantly from those expected due to constraint.

Few pairs of compounds were statistically significantly correlated for all collections. Pairs of compounds that were significantly correlated for several collections or followed consistent changes over the growing season, were considered to be of possible biological significance.

Correlations among components of needle volatile oil

Concentrations of total foliar monoterpenes varied greatly among trees. As a consequence, correlations between the amounts of individual monoterpenes were biased upwards. For this reason, there can be few conclusions about relationships among monoterpenes with correlations

of the amounts of monoterpenes per gram of tissue. The unknown high molecular weight volatile compounds were not consistently correlated with any of the monoterpenes, but were highly intercorrelated. This could indicate that they were produced from a different chemical pool of precursors.

Relationships among the foliar monoterpenes were better understood when the monoterpene composition was expressed as a percentage of the total monoterpenes. When the monoterpene composition was computed as percentages of the total foliar monoterpenes, there were strong positive correlations between tricyclene, α-pinene, and camphene (figure 17). Each compound in this group was also negatively correlated with limonene (figure 17). The correlations with α-pinene which were high at the end of the growing season, were weaker immediately after bud break. This could be the result of the high levels of α-pinene present in the buds decreasing as the needles elongated and the other compounds beginning to increase. The stronger correlations later in the growing season were representative of correlations due to foliar synthesis. Limonene and β-pinene were positively correlated (figure 17). Myrcene was negatively correlated with β-pinene, 3-carene, and limonene (figure 17).

The oxygenated compounds were not highly intercorrelated. There was a positive correlation between terpinolene and terpinen-4-ol (figure 18) and borneol and α-terpineol (figure 18). The correlation between borneol and camphor was very high when these two compounds were first detected, but they were not significantly correlated in later collections (figure 18). The correlations between borneol and bornyl

Figure 17. Changes in the correlation coefficient (r) between pairs of monoterpenes from the same organ over the growing season. Correlations were computed using 12 trees at each collection time with the monoterpene concentration expressed as a percentage of the total monoterpenes.

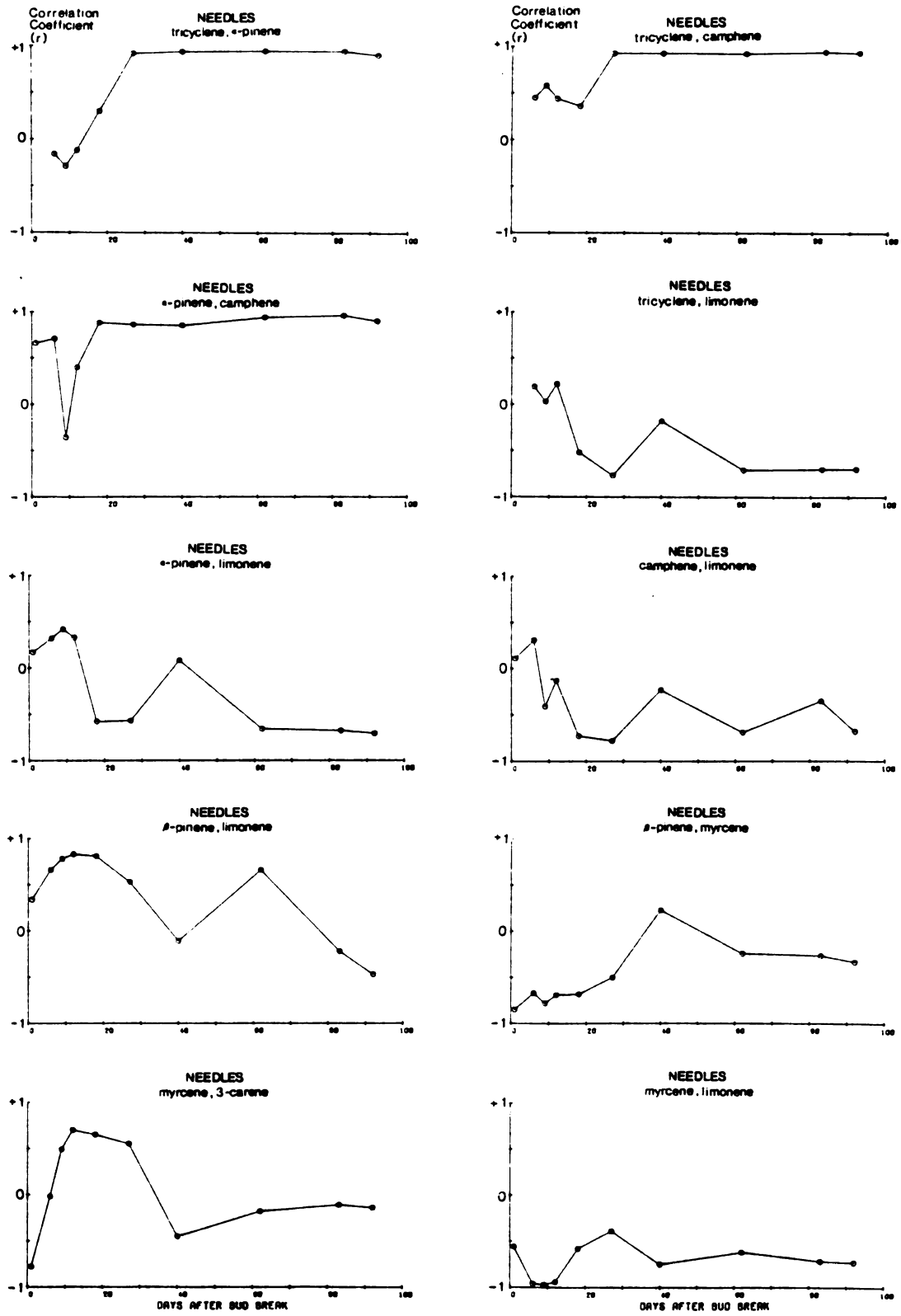

Correlation Coefficient (r)
NEEDLES
tricyclene, α-pinene
NEEDLES
tricyclene, camphene
NEEDLES
α-pinene, camphene
NEEDLES
tricyclene, limonene
NEEDLES
α-pinene, limonene
NEEDLES
camphene, limonene
NEEDLES
β-pinene, limonene
NEEDLES
β-pinene, myrcene
NEEDLES
myrcene, 3-carene
NEEDLES
myrcene, limonene
+1
0
-1
DAYS AFTER BUD BREAK

Figure 17.

Figure 18. Changes in the correlation coefficient (r) between pairs of monoterpenes from the same organ over the growing season. Correlations were computed using 12 trees at each collection time with the monoterpene concentration expressed as a percentage of the total monoterpenes.

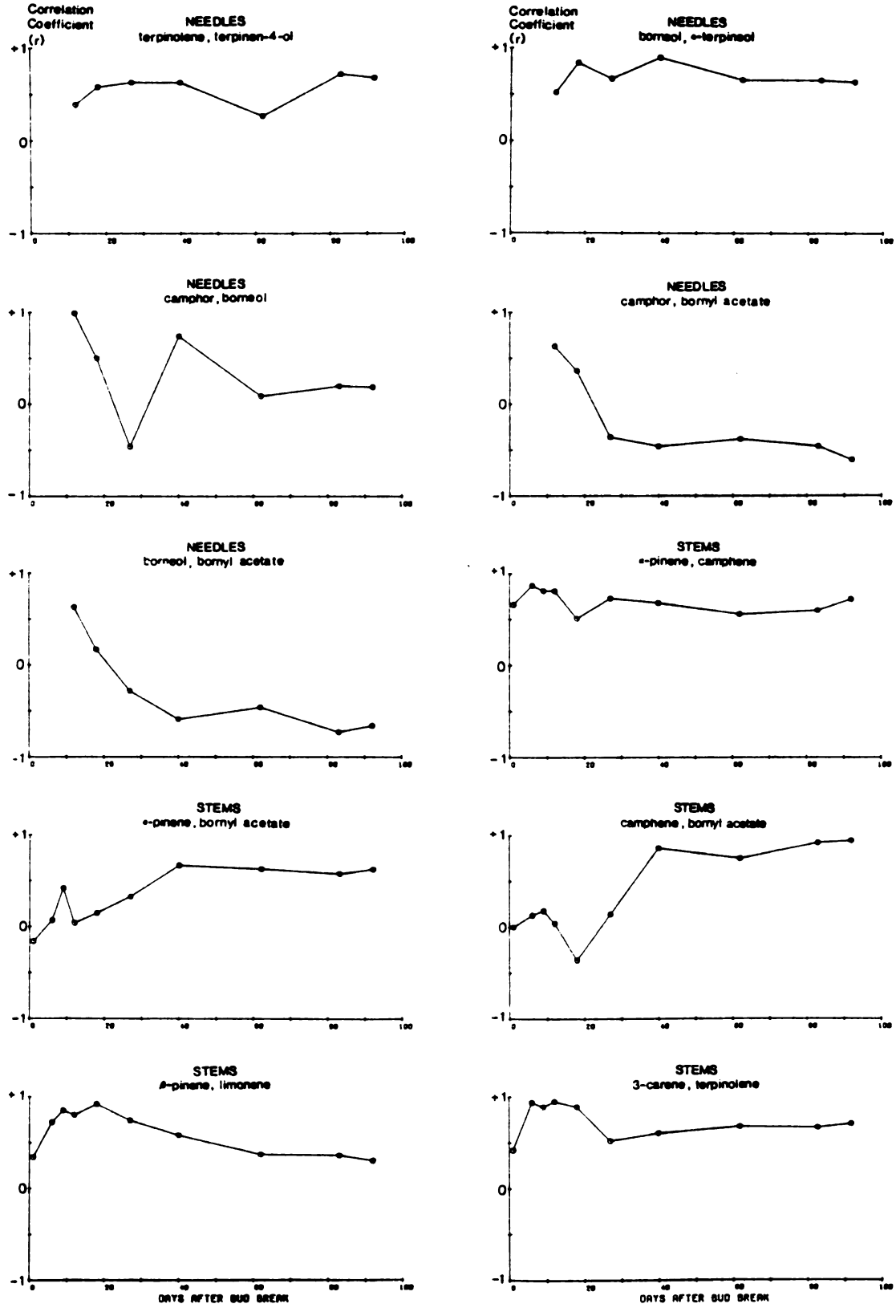

Correlation Coefficient (r)
NEEDLES
terpinolene, terpinen-4-ol
NEEDLES
borneol, α-terpineol
NEEDLES
camphor, borneol
NEEDLES
camphor, bornyl acetate
NEEDLES
borneol, bornyl acetate
STEMS
α-pinene, camphene
STEMS
α-pinene, bornyl acetate
STEMS
camphene, bornyl acetate
STEMS
β-pinene, limonene
STEMS
3-carene, terpinolene
+1
0
-1
DAYS AFTER BUD BREAK

Figure 18.

acetate (figure 18) and between camphor and bornyl acetate (figure 18) were positive when camphor and borneol were first detected and changed to negative correlations by the end of the growing season. These three compounds are proposed to be formed from the same carbonium ion (IV) in the biosynthetic scheme given in figure 19. The positive correlations among these compounds could be the result of increased levels of the common precursor. The later negative correlations could be the result of competition for the same precursor, with one compound increasing at the expense of another.

The large variations in the correlations of compounds with 3-carene and β-pinene late in the growing season are probably due to the fact that these compounds are at low levels in the foliage at this time and are subject to greater measurement error.

Hiltunen (1975) used factor analysis to determine the associations among foliar monoterpenes in Scotch pine. Compounds that were first grouped together were 3-carene, α-phellandrene, γ-terpinene, and terpinolene. The second group of compounds were tricyclene, α-pinene, camphene, β-pinene, limonene, β-phellandrene, and bornyl acetate. Myrcene was not included in either group. The composition of the volatile oil of Scotch pine is different from that of blue spruce. The compounds of the first group in Scotch pine were not present in appreciable concentrations in mature needles of blue spruce. Camphor, borneol, terpinen-4-ol, and α-terpineol were a significant proportion of the oil in blue spruce, but were not included in the groupings of Hiltunen in Scotch pine. The correlations of the compounds present in the foliage of blue spruce showed some of the same groupings of compounds as found in Scotch pine, but also some differences. The close

Figure 19. Probable pathways of monoterpenoid biosynthesis (from Zavarin, 1970 with additions from Von Rudloff, 1975a).

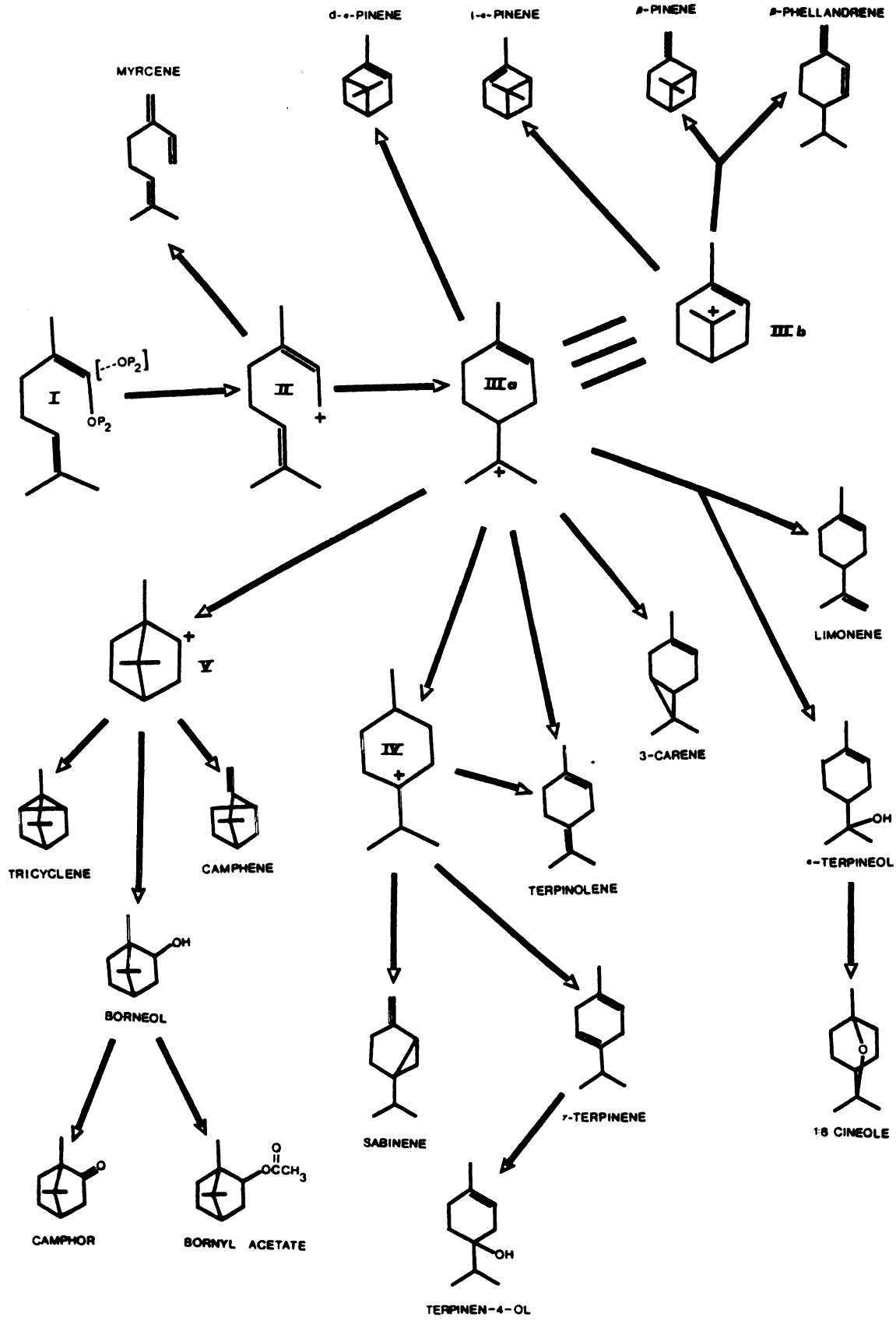

d-α-PINENE
l-α-PINENE
β-PINENE
β-PHELLANDRENE
MYRCENE
I
II
IIIa
IIIb
V
IV
LIMONENE
3-CARENE
TRICYCLENE
CAMPHENE
TERPINOLENE
α-TERPINEOL
BORNEOL
SABINENE
γ-TERPINENE
1:8 CINEOLE
CAMPHOR
BORNYL ACETATE
TERPINEN-4-OL

Figure 19.

association of tricyclene, α-pinene, and camphene was similar in both species. However, β-pinene, and bornyl acetate were not associated with these compounds in blue spruce as they were in Scotch pine.

Correlations among components of stem volatile oil

Correlations between the concentrations of the monoterpenes in stems were similar to correlations between the percentages of the monoterpenes. The unknown high molecular weight volatile compounds found in the stem were different from those found in the needles. However, the relationship to monoterpenes was the same, with the unknown stem compounds not consistantly correlated with the amounts of any of the stem monoterpenes.

Some correlations between the percentages of the stem monoterpenes were similar to those between foliar monoterpenes. Alpha-pinene, camphene, and bornyl acetate were highly positively intercorrelated (figure 18). Beta-pinene and limonene were positively correlated (figure 18). Terpinolene was positively correlated with 3-carene and γ-terpinene (figures 18 and 20). The monoterpene alcohols, terpinen-4-ol and α-terpineol, were positively correlated with each other (figure 20). The correlation between limonene and 3-carene was positive at bud break and changed to a negative correlation later in the growing season (figure 20).

Myrcene was negatively correlated with α-pinene, camphene, β-pinene, 3-carene, and limonene (figure 20). Levels of myrcene in the needles were also negatively correlated with several other needle compounds. The correlations would be in agreement with the hypothetical biosynthetic pathway given in figure 19. Carbonium (II) could be

Figure 20. Changes in the correlation coefficient (r) between pairs of monoterpenes from the same organ over the growing season. Correlations were computed using 12 trees at each collection time with the monoterpene concentration expressed as a percentage of the total monoterpenes.

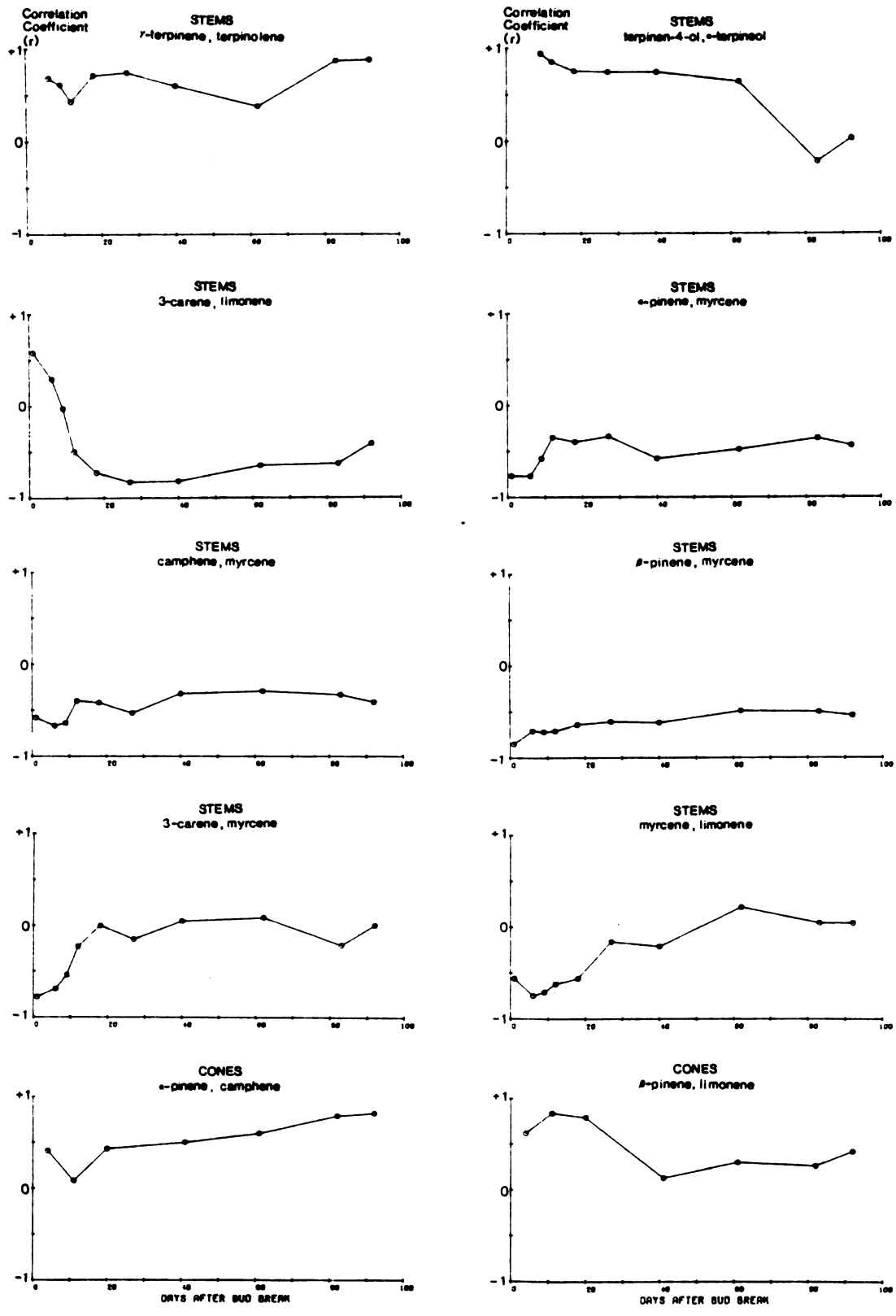

Correlation Coefficient (r)
STEMS
γ-terpinene, terpinolene
STEMS
terpinen-4-ol, α-terpineol
STEMS
3-carene, limonene
STEMS
α-pinene, myrcene
STEMS
camphene, myrcene
STEMS
β-pinene, myrcene
STEMS
3-carene, myrcene
STEMS
myrcene, limonene
CONES
α-pinene, camphene
CONES
β-pinene, limonene
+1
0
-1
DAYS AFTER BUD BREAK

Figure 20.

converted into myrcene or into carbonium ion (IIIa) which would give rise to the remaining monoterpenes. Because of this branch point myrcene would increase at the expense of the other monoterpenes and would be negatively correlated with most of the other monoterpenes.

Association of 3-carene and terpinolene has been found in studies with other species (Hanover, 1966c, 1971; Zavarin et al., 1970; Wilkinson et al., 1971; and Meier and Goggans, 1978). In agreement with the correlations found in this study, Wilkinson et al. (1971) reported myrcene to be negatively correlated with α-pinene, and both β-pinene and 3-carene to be negatively correlated with limonene in white spruce. However, they reported positive correlations between limonene and myrcene, and negative correlations between limonene and β-pinene.

Correlations among components of cone volatile oil

Most correlations between the percentages of the cone monoterpenes showed agreement with the biosynthetic scheme given in figure 19. Alpha-pinene and camphene were positively correlated as in the other tissues (figure 20). Beta-pinene was positively correlated with limonene, β-phellandrene, and α-terpineol (figures 20 and 21). All of these compounds were negatively correlated with α-pinene (figure 21), with α- and β-pinene being highly negatively correlated. Myrcene was not significantly correlated with any of the other monoterpenes.

Terpinolene and γ-terpinene were positively correlated with each other and with sabinene and terpinolene was correlated with terpinen-4-ol (figure 21). All of these compounds were hypothesized to be formed from carbonium ion (V) (figure 19). Both terpinen-4-ol and

Figure 21. Changes in the correlation coefficient (r) between pairs of monoterpenes from the same organ over the growing season. Correlations were computed using 12 trees at each collection time with the monoterpene concentration expressed as a percentage of the total monoterpenes.

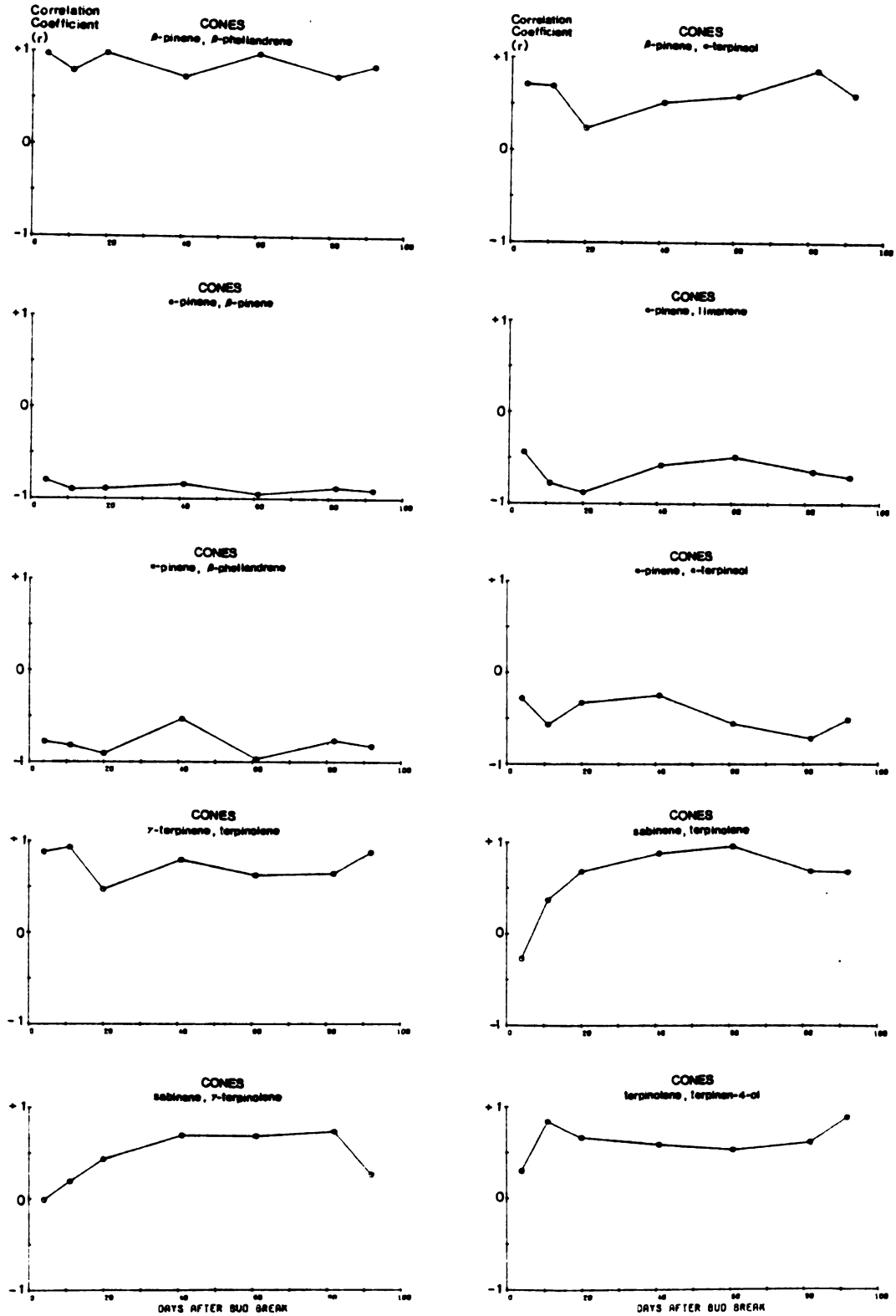

Correlation Coefficient (r)
CONES
β-pinene, β-phellandrene
CONES
β-pinene, α-terpineol
CONES
α-pinene, β-pinene
CONES
α-pinene, limonene
CONES
α-pinene, β-phellandrene
CONES
α-pinene, α-terpineol
CONES
γ-terpinene, terpinolene
CONES
sabinene, terpinolene
CONES
sabinene, γ-terpinolene
CONES
terpinolene, terpinen-4-ol
+1
0
-1
DAYS AFTER BUD BREAK

Figure 21.

γ-terpinene were present in low concentration and were subject to large amounts of error in measurement.

Similar correlations were found between amounts of monoterpenes per gram dry weight. Correlations between α-pinene and camphene were stronger on a dry weight basis than on a percentage basis and the amounts of both α-pinene and camphene were correlated with the amounts of bornyl acetate. In contrast to the correlations between percentages of monoterpenes, the correlations of the concentrations of α-pinene with β-phellandrene and of α-pinene with limonene were not statistically significant on a dry weight basis.

As with the other tissues, the unknown volatile compounds present in the cone were not consistently correlated with any of the monoterpenes.

Correlations among monoterpenes in different organs

There did not appear to be any relation between the amounts per gram dry weight of total monoterpenes in the different organs, as shown by the correlations in figure 22. The percentage of myrcene in the stem was strongly positively correlated with the percentage in the needles (figure 22). However, the amounts per gram dry weight of myrcene in these organs were not significantly correlated. Concentrations of 3-carene in the stem and cones were significantly correlated at several of the collections, but did not show any clear pattern. However, the percentage of 3-carene in these organs was positively correlated for the first part of the growing season (figure 22). The percentage of β-pinene in the stem and cone was significantly correlated

Figure 22. Changes in the correlation coefficient (r) between monoterpenes from different organs over the growing season. Correlations were computed using 12 blue spruce trees at each collection time. Concentrations for total monoterpenes were expressed as µl/g dry weight and for individual monoterpenes as a percentage of the total monoterpenes.

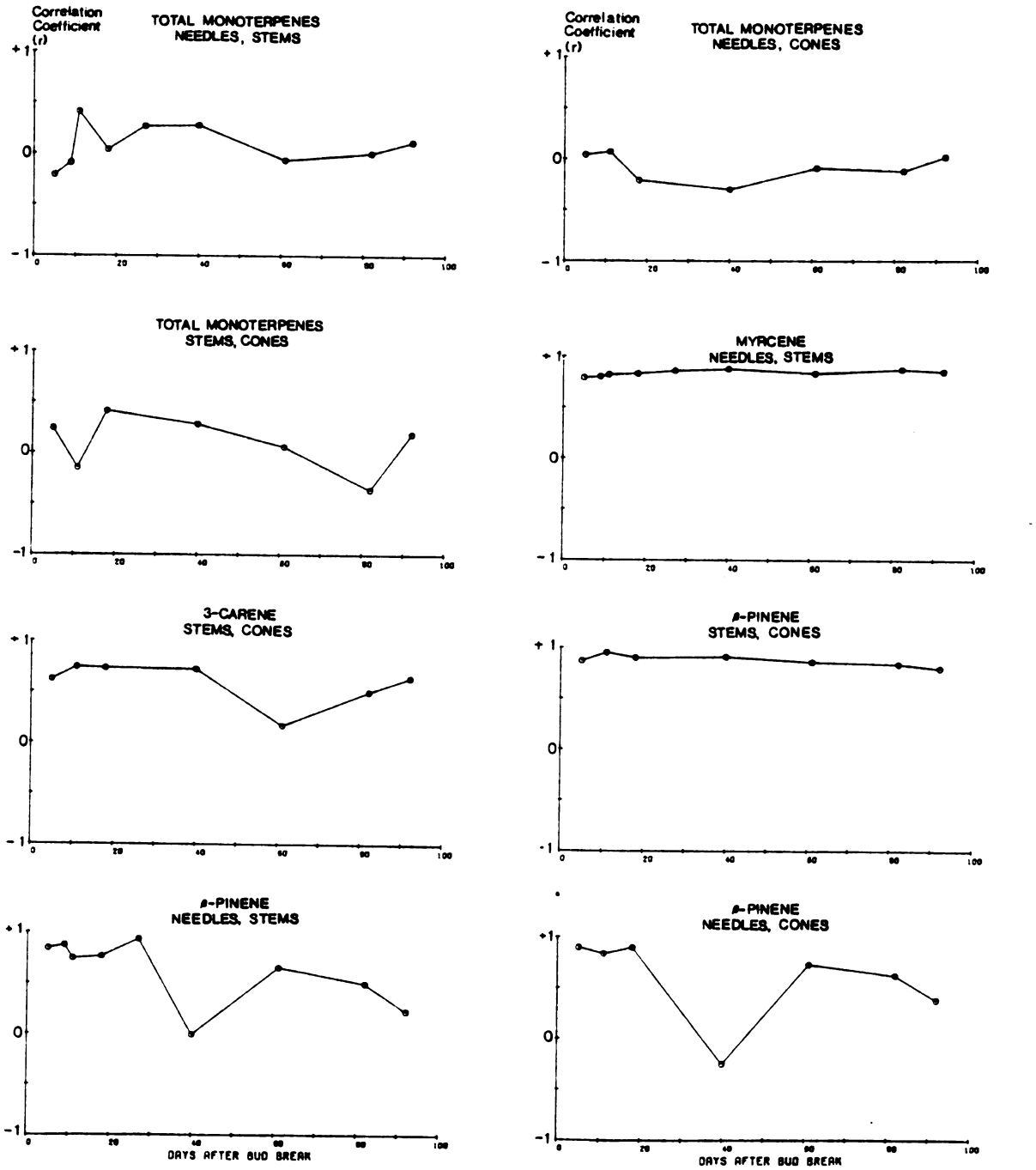

Correlation Coefficient (r)
TOTAL MONOTERPENES NEEDLES, STEMS
TOTAL MONOTERPENES NEEDLES, CONES
TOTAL MONOTERPENES STEMS, CONES
MYRCENE NEEDLES, STEMS
3-CARENE STEMS, CONES
β-PINENE STEMS, CONES
β-PINENE NEEDLES, STEMS
β-PINENE NEEDLES, CONES
+1
0
-1
DAYS AFTER BUD BREAK

Figure 22.

over the entire growing season (figure 22). The percentage of β-pinene in the needles was significantly correlated with that in both the stem and cone over the first part of the growing season (figure 22).

Relation of correlations with biosynthesis

The hypothesized biosynthetic scheme is given in figure 19 (Zavarin, 1970). It was modified to include α-terpineol, terpinen-4-ol, and 1:8 cineole from the precursors suggested by Von Rudloff (1975a). Correlations found in the different tissues of blue spruce are explained by this biosynthetic pathway with few exceptions. This biosynthetic pathway does not provide a reason for the consistent positive correlations between α-pinene and camphene or the positive correlations between β-pinene and limonene. However, Hiltunen (1975) concludes that β-pinene, β-phellandrene, and limonene are all formed from the same carbonium ion (III b). The remaining correlations could be explained as a result of changes in a precursor that would change the levels of all compounds in that portion of the pathway, and by competition for a common precursor.

Conclusions

The biosynthetic model of Zavarin (1970) agrees with most of the observed correlations in blue spruce. The means of expressing the monoterpene composition can greatly influence the correlations, especially when there is a large range in the total amounts of monoterpenes and the proportions of each compound are relatively constant. This will result in positive correlations between most compounds as occurred in needles. Correlations between percentage data can result in negative correlations that were the result of constraint. However, the

magnitude of this correlation can be estimated and deviations tested. Correlations between monoterpenes due to constraint in blue spruce were not large.

Total monoterpene concentration was not significantly correlated between the different organs. This indicates that the environmental and genetic factors which control the total concentration of monoterpenes is different for each organ. With the exception of β-pinene, myrcene, and 3-carene, the monoterpene levels were not significantly correlated between organs. This indicates that, in most cases, the monoterpene level of one organ cannot be used to predict the level in another organ.

Many correlations were significant only at certain times of the growing season. If correlations had been calculated only at the end of the growing season many would not have been detected.

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS

These studies show that much variation exists within the tree in the monoterpene composition of spruce tissues. The composition of the needles, xylem, bark, roots, and cones were very different both in the quantities of monoterpenes per gram of tissue, and in the composition of the oil. Because of these differences, care should be taken in selecting the tissue appropriate to any given study. Great variation was also found in both the amounts and the composition of the oil among trees. Differences in the quantities of oil per gram of tissue have often been overlooked in studies of tree resistance to pests. Trees may be more resistant to an insect, pathogen, or a browsing animal because of variations in both the composition and the amounts of the monoterpenes. Tests should be conducted to determine if both the quantity and the composition of the oil are involved in resistance.

Monoterpene content also varies in respect to position in the crown. In xylem the yield of oil increased in tissues of the same age with increasing height in the tree. The yield of oil was highly correlated with the proportion of resin canals to other tissues in the xylem. This increase in the proportion of resin canals in the top of the tree was not due to an increase in the cross sectional area of the individual xylem resin canals, but to a higher density of resin canals. Not only did the yield of total xylem monoterpenes vary with height in the tree; but composition of the volatile oils varied also. The yield

of foliar monoterpenes also differed with position in the crown but the differences were the reverse of the xylem. That is, the yield of total foliar monoterpenes differed little between the top and middle portions of the tree, but almost doubled in the bottom of the tree. The physiological or environmental causes of the differences in monoterpenes with height in needles are not known. The differences in the foliage composition could not be accounted for by the volatility of the compounds, because the compounds present in the highest proportions in the top of the tree were the more volatile compounds. The yield of volatile oil was very highly correlated with the proportion of the resin canals to all other needle tissues. The increases in the volume of the resin canals in the bottom of the tree were not simply a uniform increase through the entire needle, although the volume of resin canal in the bottom of the tree was larger in all portions of the needle. Needles from the bottom of the tree had a higher proportion of the total resin canal at the tip of the needle than did needles from the top of the tree. This difference could be important in insect behavior. The yield of total bark monoterpenes did not vary significantly with position in the crown. There were few differences with height for either the yield or percentage of individual monoterpenes. These within-tree differences in amounts of monoterpenes have not been taken into account in many studies of tree resistance, chemosystematics, and genetics. Bark monoterpenes may be more suitable for some studies, because of the large among-tree variation and the smaller amounts of within-tree variation than in either foliar or xylem monoterpenes. Variation was also associated with stem samples of different ages. Such variation may be present in other tissues and must be taken into

account in planning any monoterpene-related studies.

In the xylem, differences between the yield of oil from the top and middle of the tree were similar to the differences between the middle and bottom of the tree. In the needles there was little difference in yield between the top and middle of the tree, but a very large difference between the middle and bottom. The position in the crown where this transition in the amounts of oil per gram of tissue takes place may provide information about the factors involved in causing this difference. This transition may take place at a different position in the crown or be absent in smaller trees than those used in these studies.

The behavior of an insect or a browsing animal may be affected not only by the composition and amounts of monoterpenes, but also by differences in the emission of volatiles among the trees. Trees with higher amounts of monoterpenes per gram of tissue may release more volatiles to the atmosphere and thereby affect pests. Differences between the amounts of volatiles released from the top and bottom of the tree could also have an effect. The amounts and rates of emission of volatiles probably relates to the anatomy of the organs. The effect of these differences on the behavior of pests should be tested.

Organs differed in seasonal development of the yield of volatile oil. The needles and the stem both increased in the amounts of monoterpenes per gram dry weight tissue and then maintained this level through the remainder of the growing season. The increase in the amounts of monoterpenes occurred at different times in the needles and stem. The stem monoterpene content, on a dry weight basis, increased

immediately after bud break and reached the maximal level before cessation of stem elongation. The needles did not increase in amounts of monoterpenes on a dry weight basis until needle elongation had ceased. The amounts on a dry weight basis then increased rapidly. The cone monoterpene content increased after bud break, decreased after the cones became pendant, then increased again from the time the seeds were viable to the end of the growing season. When the amounts of total monoterpenes were calculated per cone, the increase over the growing season was almost linear. The amounts of monoterpenes per cone continued to increase after the cones had reached their maximal weight. The cone monoterpenes did not reach a stable level at any time of the growing season and for this reason would be difficult to use in systematic and genetic studies.

Large amounts of unknown high molecular weight volatile compounds were detected in the stem and cones. These compounds varied between trees and over the growing season. The identity of these compounds and their genetic control should be investigated.

Developing tissues changed rapidly in monoterpene composition. By applying radioactive tracers at specific stages of development to specific tissues the compounds that become labelled may provide information on the biosynthetic pathways that are active at that time and the biosynthetic relationship between compounds.

Correlations between different monoterpenes over the growing season agreed very well with the proposed biosynthetic scheme of Zavarin (1970). However, the correlations between β-pinene and limonene and the high correlations between α-pinene and camphene in all three tissues

could not be explained by this model. The correlation between α-pinene and camphene was high in all three tissues even though camphene was present in only 3% of the amounts of α-pinene in the stem, 2% in the cone, and 1.7 times the amounts of α-pinene in the needles. There obviously were large shifts in the biosynthetic pathways involved, but the correlations remained high.

In Douglas-fir the resin canals are one of the first tissues to differentiate (Owens, 1968). In blue spruce, monoterpenes were detected in the first collection of needles after bud break, but monoterpene levels did not appear to increase on a dry weight basis until after elongation had ceased. The relationship between the development of the resin canal and the production of volatile oils in blue spruce needles is not clear. The resin canals of blue spruce may develop more slowly than in Douglas-fir, or the resin canals may be present, but not accumulate volatile oils early in the growing season.

The percentage of some of the monoterpenes showed groupings at certain levels. For example, the percentage of bornyl acetate in the needles showed two distinct groups. These differences may be under simple genetic control, possibly single gene control. However, there have been few genetic studies of foliar monoterpenes. In addition to differences in the composition of the oil, there were large differences in the yield of total oil in the needles. These differences did not show distinct groupings, but may be under genetic control. The yield of oil may be controlled by several genes and may have large environmental effects. The possibility of genetic control of both the quantities of total monoterpenes and the composition of the oil should be tested.

The means of expressing data can greatly affect conclusions. Variation of foliar tricyclene provides one example. When the level of tricyclene was expressed in amounts per gram dry weight of tissue there was a statistically significant difference at the one percent level with position in the crown, but no significant difference among trees. When tricyclene was expressed as a percentage of the total monoterpenes the differences were statistically significant among trees, but not significantly different with position in the crown. Contradictory results could be obtained with the two ways of expressing the data, because there were large differences with position in the crown and among trees in the total yield of monoterpenes. Contradictory results could be obtained with correlations also. The amounts of myrcene and limonene were positively correlated in the foliage ($r = +.79$ $p \leq .01$) at the end of the growing season, while the percentages of these two compounds with the same data were negatively correlated ($r = -.73$ $p \leq .01$). The large differences in yields of monoterpenes in the needles of the trees in these studies result in many positive correlations among compounds when the amounts of these compounds are expressed as yields of individual monoterpenes. The results of percentage correlations are biased also, but in the opposite direction. Because the total of the percentages must be 100%, there is a correlation due to constraint, although this was not a problem with the monoterpenes in this study.

There is no single basis for expressing monoterpenes in developing tissue to describe accurately the changes in monoterpene levels. Developing tissues are expanding very rapidly and the dry weight to

fresh weight ratio changes rapidly. Changes in the yield per gram fresh weight are sensitive to changes in the water status early in the growing season. In order to determine yields on a dry weight basis, the fresh and dry weights of a sample must be determined in duplicate samples and the yields converted. This conversion is an additional source of error. Both the fresh weight and the dry weight of the biosynthetic unit change rapidly over the season, so the basis for the calculations is constantly changing. The amounts of a compound expressed as a percentage is not a stable basis either. The percentage of a compound may change as the result of changes in other compounds in the absence of a change in the compound of interest. Changes in the percentage of a compound do not provide information on changes in the absolute level of the compound of interest.

Some problems associated with seasonal changes are illustrated by cone myrcene. The amounts of cone myrcene per gram of tissue increased between the second and third collection times, on both a fresh weight and a dry weight basis. However, myrcene as a percentage of the total monoterpenes decreased over the same time period. From the third to the fourth collection time the amounts of myrcene decreased on a dry weight basis and remained relatively constant on a fresh weight or percentage basis. Over the remainder of the growing season the percentage and amounts of myrcene on a tissue basis decreased. When the amounts of myrcene in a cone were calculated and compared with the other measures, there is a clearer view of the changes occurring. The amounts of myrcene in the cone increased for the first 40 days after bud break and then remained relatively constant for the

remainder of the growing season. During the first 40 days of the growing season the cones increased in fresh weight. The rate of increase in weight of the cone was greater than the increase in the amounts of myrcene in the cone, so the amounts of myrcene per gram decreased. At the same time other monoterpenes were being produced at a more rapid rate than myrcene, so the percentage of myrcene decreased. The results of calculating the amounts of myrcene per cone indicated that there was no decrease in the amounts of myrcene, and that the apparent decrease was due to changes in other factors. Similar analysis could be conducted on foliar monoterpenes to determine if there was a loss of some of the monoterpenes which appear to decrease rapidly in the early part of the growing season. The changes shown by myrcene demonstrate that the data must be examined carefully by as many means as possible, and the methods must be suited to the particular purpose of the study.

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