Acta Bot. Neerl. 27 (3/4), August 1978, p. 229–238.

INTRA-SPECIES VARIATION IN THE CHEMICAL COMPOSITION OF NEEDLES OF LARIX LEPTOLEPIS

G. J. NIEMANN and W. J. BAAS

Botanisch Laboratorium, Utrecht

Key word index: Larix leptolepis; Pinaceae; flavonoids; wax constituents; intra-species variation.

SUMMARY

Leaves of *Larix leptolepis* trees and seedlings were examined for flavonoids and for wax constituents by high-performance liquid chromatography or by gas chromatography. Within the species, due to differences in genotype, there is a great variability in the chemical composition of the flavonoids but less in that of the wax constituents of the needles.

1. INTRODUCTION

Leaf constituents of species in the family of the Pinaceae have often been investigated because of their economic importance (soap and perfume industry), their possible relation to resistance (SMELYANETS 1977) or their possible use as taxonomic markers. Especially the volatile leaf oil components of a number of evergreen species were investigated for the purpose of distinguishing between species and for studying hybridization between species (*Picea* – VON RUDLOFF 1967 a and b, 1975 a; VON RUDLOFF & HOLST 1968; *Pinus* – MASON-JOYCE et al. 1972). Phenols have been correlated with resistance to insects such as the spruce gall aphid (*Picea abies* needles, TJIA & HOUSTON 1975) or to fungi such as *Guignardia laricina*, the cause of shoot blight of larch trees (NOMURA & KISHIDA 1978 a and b, in press).

In all these investigations on leaf constituents care has to be taken because of the rapid turnover of compounds within these plant organs. Thus, considerable variation was found in the volatile leaf oil components (VON RUDLOFF 1972, 1975 b and c, 1978; ZAFRA & GARCIA-PEREGRIN 1976). Also, in leaf wax of *Picea pungens* (VON RUDLOFF 1959), of *Pinus sylvestris* (SCHÜTT & SCHUCK 1973), and of *Picea abies* (SCHUCK 1976) in fatty acids and paraffins a great variation occurs according to the season, the leaf age and even the exposition of the branches. For flavonoids similarly large seasonal variations in synthesis (*Picea* – DITTRICH 1970), composition and concentration were found (*Picea* – FORREST 1975 a; *Larix* – NIEMANN 1976, and NOMURA 1976). In addition to seasonal variation, VARAKSINA (1973) also found a diurnal variation in the quantities of five flavonoids in *Larix sibirica* needles.

The geographic variation was studied by WELLENDORF et al. (1971) (unidentified phenols in *Picea abies*), by LA ROI & DUGLE (1968) (unidentified com-

pounds in needles of *P. glauca* and *P. engelmannii*), and by FORREST (1975 b) (polyphenols of *P. sitchensis* needles). FORREST (1975 b) also found a marked difference in the polyphenol composition of needles of plants from two different clones.

In *Larix* needles a rich array of phenolic glycosides was observed in all species investigated (NIEMANN 1969–1976; NIEMANN et al. 1971–1977; MEDVEDEVA et al. 1974; TJUKAVKINA et al. 1975). From the needles of *L. leptolepis*, in addition to a number of glycosides and esters of hydroxybenzoic- and cinnamic acids (NIEMANN 1971, 1973 a, 1976) about twenty flavonoids were isolated and identified (NIEMANN 1973 b, 1974, 1976; NOMURA & MUTO 1976). Less work has been done on the leaf lipids; GOAD & GOODWIN (1967) isolated the sterols β-sitosterol and campesterol, the 4-α-methylsterols cycloeucalenol, 24-methylene-lophenol and 24-ethylidene-lophenol, and the triterpenols cycloartanol and 24-methylene-cycloartanol from the newly opened leaves of *L. decidua*. JAMIESON & REID (1972) found some saturated and unsaturated fatty acids in needles of *L. leptolepis* and *L. decidua* with a seasonal variation in concentration.

In a previous publication (NIEMANN 1976) attention was paid to the large seasonal variation in a number of main flavonoids isolated from needles of one tree of the species *L. leptolepis*. At present we are interested in the possible variation of phenols as well as terpenoid wax constituents in needles taken from different plants of the same species. Although some attention has also been paid to components of trees growing at different sites, mainly seedlings of *L. leptolepis* growing under the same conditions have been used, to avoid effects of differences in environment.

2. MATERIAL AND METHODS

Needles were picked from two-year old seedlings grown in the greenhouse. The seed was obtained from the Dutch State Forestry Service at Arnhem, The Netherlands, and was of West-German origin. In addition, needles were collected from three fully-grown trees at Austerlitz, from one tree in the arboretum "Schovenhorst" at Putten, both in The Netherlands, and from a tree growing at Sapporo, Japan. In the latter cases needles were picked in August.

The seedling needles were either used while fresh or after being kept for a short period in a deepfreezer. The wax components were sampled by treating the needles twice for 20 seconds with chloroform. Hereafter, the needles were extracted with acetone-water as described before (NIEMANN & KOERSELMAN-KOOY 1977) which leads to a butanol extract containing practically all the flavonoids. Needles of older trees were obtained freeze-dried (Austerlitz/Putten) or oven-dried (Sapporo), therefore wax constituents were not collected. Both the chloroform and butanol extracts were evaporated to dryness and dissolved in methanol for a first screening by high-performance liquid chromatography (HPLC). The chloroform extracts were only partly soluble in methanol. The methanol extracts were analysed by HPLC with a Dupont

830 chromatograph on a Zorbax ODS column, 25 cm × 4.6 mm ID, at 50°C and a pressure varying from 1100–3000 psi (flow from 0.6–1.2 ml/min.). For the wax extracts methanol with 0.1% of phosphoric acid was used, for the phenol fractions a concave (2) gradient programmed from 45% methanol-water to 100% methanol, again with 0.1% of phosphoric acid. Detection occurred both with UV at a fixed wavelength of 254 nm, and with a Dupont 837 spectrophotometer at 215 nm for the lipid fraction and at 360 nm for the flavonoids. As internal standard uvaol was used for the lipid fraction, and vitexin (V) for the phenols. In addition to vitexin, the phenol fractions were also co-chromatographed with kaempferol-3-glucoside (KG) and kaempferol-3-(p-coumarylglucoside) (KCG). In later analyses, the column was provided with a 10 cm chrompack pre-column with Co:Pell ODS to avoid column deterioration; a drop in separation quality was unavoidable.

Both total lipid extracts and the methanol soluble part of them, were also analysed by gas-liquid chromatography (GLC) on a glass column, 1.20 m × 4 mm ID on Varaport-30 coated with 3% SE 30, temperature programmed from 240 to 320°C, or isotherm at 240°C. For further analysis the total lipid fraction was separated by alumina column chromatography with increasing concentrations of diethyl ether in ligroin, into five fractions (0%, 10% – esters, 30% – triterpene alcohols, 40% – sterols, and 100% ether), which were investigated by GC-MS in the untreated state and/or after saponification (BAAS 1977).

3. RESULTS AND DISCUSSION

Needles of Austerlitz tree number 3 were extracted by different persons in fourfold, those of the Japanese tree (no 5) in eightfold. All twelve extracts were investigated both by paper-chromatography and by HPLC. No differences between the four extracts of the Austerlitz tree, or between the eight extracts of the Japanese one were found. When the Austerlitz needle extracts were compared with the Japanese, on paper only slight differences, seen as colour variation, were present, but with HPLC analysis a much larger difference became apparent; this can be seen in *fig. 1* (no 3 and 4) which gives the chromatograms of three different Austerlitz trees (nos 1–3), the Japanese one (no 4) and the Putten tree (no 5). Only in the case of two trees, nos 1 and 2, was an almost similar flavonoid pattern obtained; the others were completely different, especially in their relative concentrations. In some cases there was an almost total absence of the vitexin-like compounds. Of course, it is not clear whether climatological or genotype variation must be held responsible for the differences found.

Not all compounds were identified, but in view of the former needle analyses of different species of larch (NIEMANN 1971–1976), it is highly probable that the same compounds are present and that the variability is caused by quantitative rather than qualitative differences.

Of the seedlings eight plants, randomly selected, were analysed for both

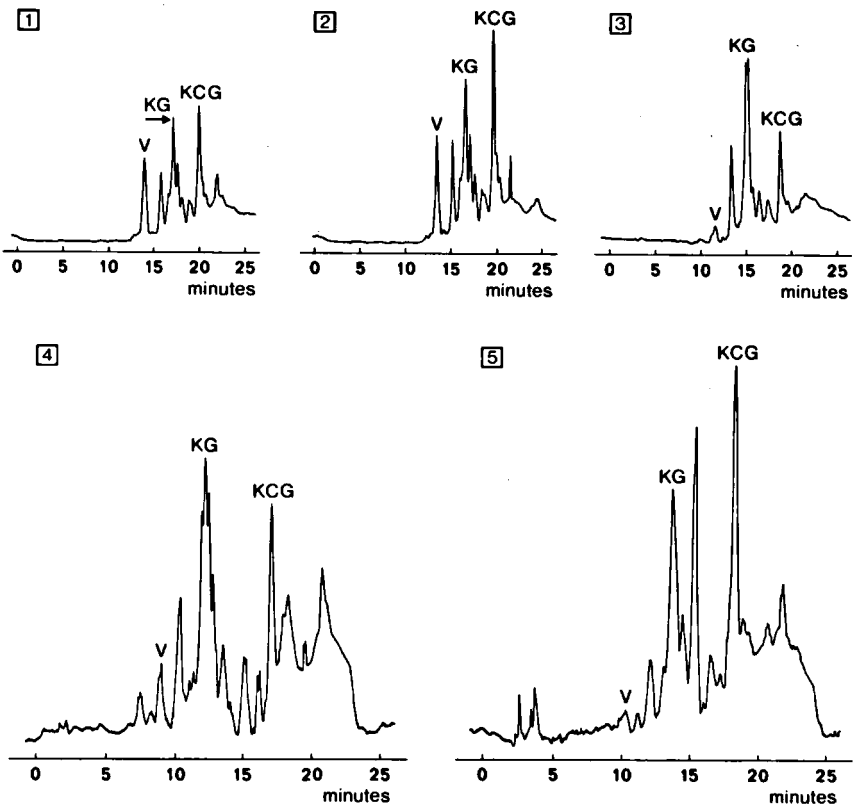

Fig. 1. HPLC separation of flavonoids from needles of *Larix leptolepis* on Zorbax ODS. Needles picked in August from trees growing at Austerlitz, The Netherlands (nos 1, 2 and 3), at Sapporo, Japan (no. 4), and at Putten, The Netherlands (no. 5). V = peak which co-chromatographs with vitexin, KG with kaempferol-3-glucoside and KCG with kaempferol-3-(p-coumarylglucoside).

lipophilic compounds and phenolics. The methanol-soluble part of the total lipid fractions was investigated both by HPLC and GLC. In *fig. 2 A* and *B* two extreme types of variation found are shown; all kinds of intermediate forms occur. In the GLC chromatograms only a slight variation was found, with HPLC, however, rather a high variation occurred in the triterpenol area (NIEMANN & BAAS 1978) at peaks nos 5 to 7. By further GLC analysis of the eluted compounds HPLC peak 7 co-chromatographs with the high peak at 12 minutes, and no. 6 with that at 24 min., and with triterpenols such as 24-methylene-cycloartanol. Further GLC analysis of the chloroform-soluble part of the lipid fractions is tabulated in *table 1*, from which only a slight variation in the quantitative division becomes apparent. *Fig. 3* gives the GLC chromatogram of the total lipid fraction of needles of plant no. 8. Column 2 of *table 1*

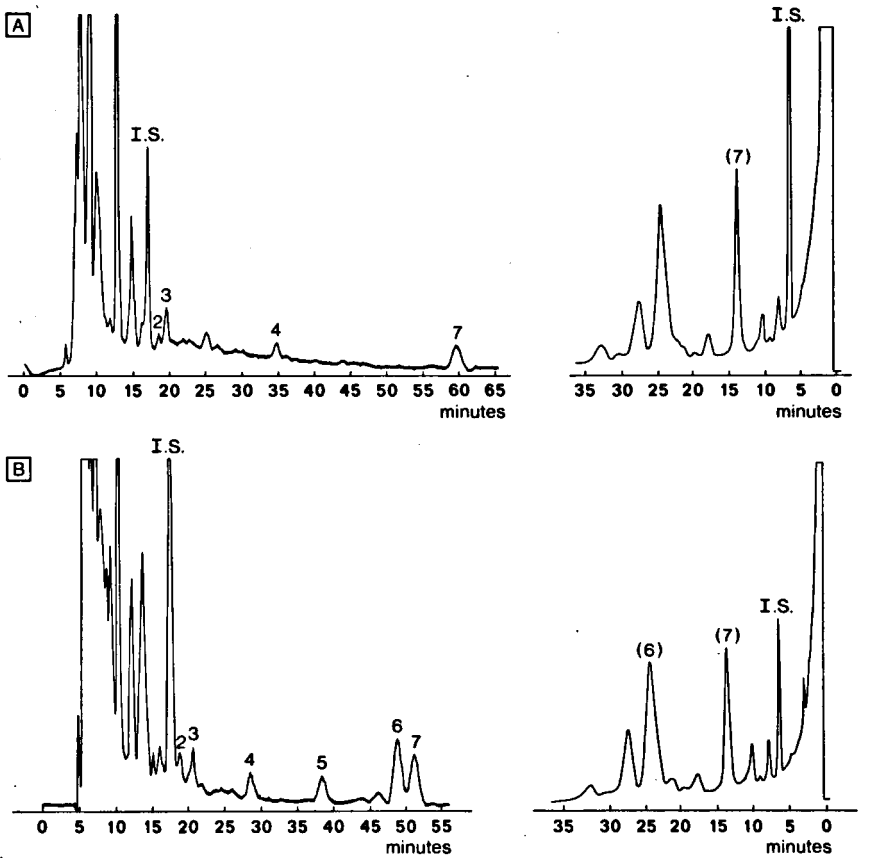

Fig. 2. Separation of the methanol-soluble lipophilic needle compounds of two *Larix leptolepis* seedlings (A and B) by HPLC on Zorbax ODS (left) and by GLC on SE 30 (right).

shows the fraction in which some of the major peaks occur on subdivision by alumina column chromatography.

Although in the total lipid fraction co-chromatography indicated the possible presence of traces of β-sitosterol and campesterol, these compounds were not found in the sterol fraction. Thus, contrary to the investigation by GOAD & GOODWIN (1967) of needle extracts from *Larix decidua*, the free sterols were not found in the wax of *L. leptolepis* needles. Saponification of the 10% fraction, on the other hand, yielded a new peak indicating a comparatively high concentration. RT and mass spectrum proved this compound to be identical with β-sitosterol, which therefore occurs as an ester instead of in the free form. The main peak in the 10% fraction (peak no. 3 of the total lipid fraction, *fig. 3*) is unsaponifiable; main fragments in its mass spectrum were $C_{20}H_{41}O$ (*m/e* 297) and $C_{29}H_{58}$ (*m/e* 406) which together with the peaks at

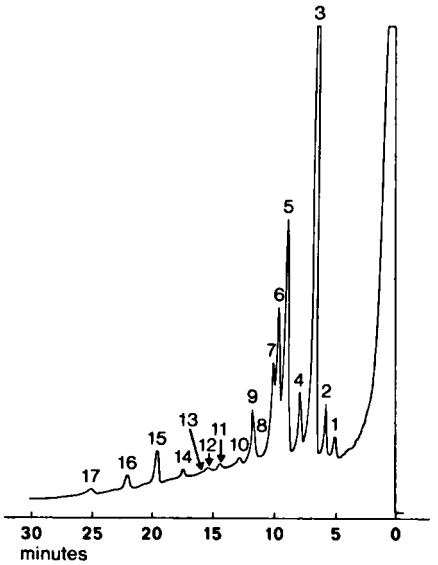

Fig. 3. GLC analysis of chloroform-soluble needle compounds from *Larix leptolepis* seedlings on 3% SE 30, temperature programmed from 240 to 320°C.

Table 1. Variation in the chloroform-soluble fraction of the needle wax of eight seedlings of *Larix leptolepis*, screened by GLC on 3% SE 30, temperature-programmed from 240°–320°C, figures in % of total peak area.

Compound	Fraction*	Seedling Number							
		1	2	3	4	5	6	7	8
1	30%	0.9	1.0	1.0	1.0	0.9	0.8	0.8	0.3
2	30%	2.0	2.5	1.7	1.5	0.5	1.4	1.4	2.6
3	10% (30%)	57.8	56.1	55.0	62.1	62.5	56.2	66.2	51.7
4	(10%) 30%	4.0	4.0	3.0	1.9	1.9	3.8	1.8	4.5
5	100%	14.4	15.3	17.7	13.5	14.4	16.6	3.1	17.1
6	10%/30%	7.7	6.2		6.6	5.5	2.0		8.4
6	10%/30%	7.7	6.2	8.4	6.6	5.5	2.0	9.2	8.4
7	10%	5.8	5.8		3.4	3.3	2.1		6.4
8	–	–	0.9	–	0.7	0.8	0.4	–	–
9	0%	3.3	3.3	4.2	2.9	4.3	4.4	2.0	2.8
10	–	0.2	1.1	1.8	1.2	1.2	1.8	0.8	0.3
11	0%	0.1	0.3	0.6	0.7	0.2	0.3	0.2	0.2
12	–	–	0.1	0.6	0.2	0.2	0.2	0.1	–
13	–	–	0.2	0.1	–	0.1	0.1	–	–
14	–	0.5	0.3	0.4	0.5	0.4	0.7	0.5	0.6
15	0%	1.6	1.0	1.7	1.3	1.1	1.7	1.7	2.2
16	0%	0.7	0.5	1.0	0.6	0.4	0.6	1.1	1.0
17	0%	0.6	0.6	0.5	0.4	0.2	0.5	0.7	0.7

* Fraction or fractions in which the peak is found after alumina column chromatography.

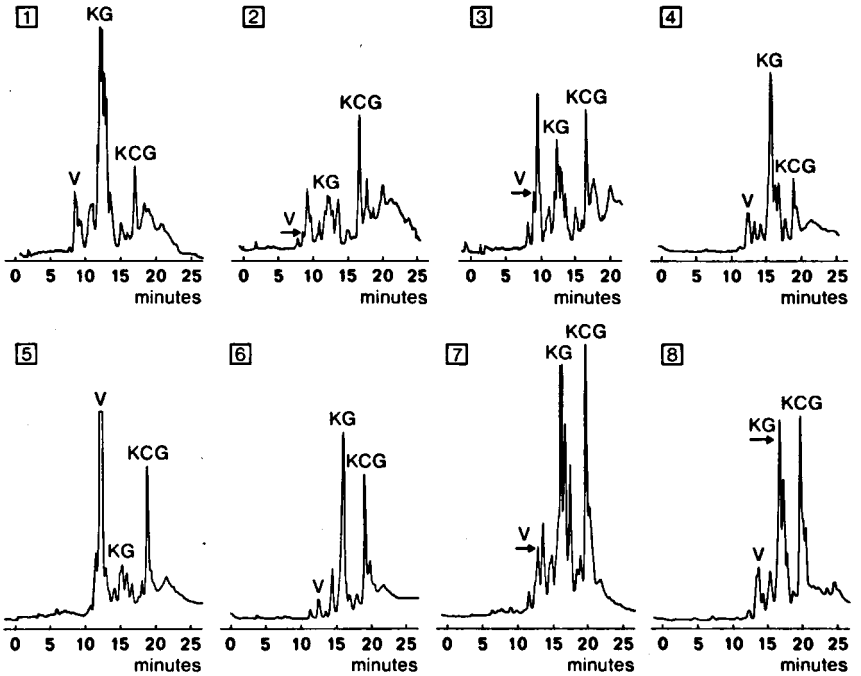

Fig. 4. HPLC separation of needle flavonoids of *Larix leptolepis* seedlings on Zorbax ODS. V = peak which co-chromatographs with vitexin, KG with kaempferol-3-glucoside, and KCG with kaempferol-3-(p-coumarylglucoside).

m/e 378, 278 and 157 and a very weak molecular ion peak at *m/e* 424 indicates $C_{19}H_{39}CH(OH)C_9H_{19}$ or 10-nonacosanol (BERI & LEMON 1970).

Of the 30% fraction some of the minor components co-chromatographed with β-amyrin, cycloartanol and cyclolaudenol respectively.

In *fig. 4* the HPLC chromatograms of the eight butanol fractions of the seedling needles extracts are collected. This figure shows the tremendous variation in flavonoid composition in different genotypes of the species *L. leptolepis*. Actually, not one of the eight extracts is similar to any other. Since propagation of *Larix* in general only occurs by means of seed, the situation, demonstrated in seedling variation must be present throughout the whole *L. leptolepis* population. A similar effect was found during a simultaneous investigation by NOMURA & KISHIDA (1978 a – personal communication) of the phenolics of the annual shoots of eight different clones of *L. leptolepis* in which a large inter-clonal variation was demonstrated for the compounds eriodictyol, epiafzelechin, catechin, taxifolin (= dihydroquercetin) and taxifolin-3'-glucoside.

Studies on the effects of environmental factors or of provenance in connection with phenolics have often been done using only two to three trees (FOR-

REST 1975b, WELLENDORF et al. 1971). In the case of *L. leptolepis* these studies on phenolics of individual plants may give completely wrong results because of the variability within the species. Unless one is sure about the genetic homogeneity of its plant material, the use of clonal material or of statistically justified numbers of plants is essential.

The interspecies comparison within the genus *Larix* given by us last year (NIEMANN & KOERSELMAN-KOOY, 1977) must now be amended, since in all cases only one tree of each species was used for investigation. Actually, the variation found in that investigation does not differ essentially from that found within the species *L. leptolepis*.

ACKNOWLEDGEMENT

The authors are grateful to Judith Koerselman-Kooy for her skilful assistance, to Frouke Gerards-Kuyer for the drawings, to Mr. Kazutaka Nomura for the Japanese larch needles, to twelve undergraduate students for their contribution and to Miss S. M. McNab for the correction of the English text.

REFERENCES

BAAS, W. J. (1977): Triterpenes in latex of Euphorbia pulcherrima. *Planta med.* **32:** 1–8.

BERI, R. M. & H. W. LEMON (1970): Chemical examination of the wax from needles of black spruce (Picea mariana) and balsam fir (Abies balsamea). *Can. J. Chem.* **48:** 67–69.

DITTRICH, P. (1970): *Untersuchungen über den Umsatz sekundärer Pflanzenstoffe in den Nadeln von Picea abies* L. Thesis, Ludwig, Maximulans Universität, München.

FORREST, G. I. (1975a): Polyphenol variation in Sitka spruce. *Can. J. For. Res.* **5:** 26–37.

— (1975b): Variation in polyphenol content within and between Sitka spruce provenances at different sites. *Can. J. For. Res.* **5:** 46–54.

GOAD, L. J. & T. W. GOODWIN (1967): Studies on phytosterol biosynthesis: the sterols of Larix decidua leaves. *European J. Biochem.* **1:** 357–362.

JAMIESON, G. R. & E. H. REID (1972): The leaf lipids of some conifer species. *Phytochemistry* **11:** 269–275.

LA ROI, G. H. & J. R. DUGLE (1968): A systematic and genecological study of Picea glauca and P. engelmannii using paper chromatograms of needle extracts. *Can. J. Bot.* **46:** 649–687.

MASON-JOYCE, N., A. T. PROVEAUX & R. V. LAWRENCE (1972): Composition of pine needle oil. *J. Chromatographic Science* **10:** 590–592.

MEDVEDEVA, S. A., N. A. TJUKAVKINA & S. Z. IVANOVA (1974): Phenolic extractives from needles of the Siberian larch. *Khimia drevjesni (Riga)* **15:** 144–152.

NIEMANN, G. J. (1969): Phenolic glucosides from needles of Larix laricina. *Phytochemistry* **8:** 2102–2103.

— (1971): Phenolic constituents from Larix needles III: Benzoic and cinnamic acids of L. leptolepis. *Acta Bot. Neerl.* **20:** 397–400.

— (1972): Phenolics from Larix needles IV. Constituents of L. larcina. *Acta Bot. Neerl.* **21:** 549–552.

— (1973a): Phenolic glucosides from needles of Larix leptolepis. *Phytochemistry* **12:** 723–724.

— (1973b): Flavonoids from needles of Larix leptolepis. *Phytochemistry* **12:** 2056.

— (1974): Phenolics from Larix needles VIII. Flavonoids of L. leptolepis. *Planta med.* **26:** 101–103.

— (1975a): Main flavonoids in needles of Larix decidua. *Phytochemistry* **14:** 1436–1437.

— (1975b): Phenolics from Larix needles X. Flavonoids of L.gmelinii. *Acta Bot. Neerl.* **24**: 65–68.
— (1975c): Acylated flavonol glycosides from Larix needles. *Phytochemistry* **14**: 1437–1438.
— (1976): Phenolics from Larix needles XII. Seasonal variation of main flavonoids in leaves of L. leptolepis. *Acta Bot. Neerl.* **25**: 349–359.
— & W. J. BAAS (1978): High-performance liquid chromatography of triterpenols. *J. Chromatogr. Sci.* **16**: 260–262.
— & R. BEKOOY (1971): Flavonoid constituents from Larix needles. *Phytochemistry* **10**: 893.
— & J. W. KOERSELMAN-KOOY (1977): Phenolics from Larix needles XIII. Analysis of main Larix flavonoids by high-pressure liquid chromatography. *Planta med.* **31**: 297–301.
NOMURA, K. (1976): Studies on the constituents of the annual shoots of Larix leptolepis Gordon (IV) Seasonal changes of the phenolic compounds. *J. Jap. For. Soc.* **58**: 379–382.
— & K. MUTO (1976): Studies on the constituents of the annual shoots of Larix leptolepis Gordon (I) Kaempferol-3-0-glucoside in the leaves during the growing season. *J. Jap. For. Soc.* **58**: 86–91.
— & A. KISHIDA (1978a): Studies on the constituents of the annual shoots of Larix leptolepis Gordon (VI) Seasonal changes of phenolic compounds in relation to shoot blight resistance. *In press.*
— &—, (1978b): Studies on the constituents in the annual shoots of Larix leptolepis Gordon (VII) Significance of phenolic compounds as a marker of shoot blight resistant larch. *In press.*
RUDLOFF, E. VON (1959): The wax of the leaves of Picea pungens (Colorado spruce). *Can. J. Chem.* **37**: 1038–1042.
— (1967a): Chemosystematic studies in the genus Picea (Pinaceae). I. Introduction. *Can. J. Bot.* **45**: 891–901.
— (1967b): Chemosystematic studies in the genus Picea (Pinaceae). II. The leaf oil of Picea glauca and P. mariana. *Can. J. Bot.* **45**: 1703–1714.
— (1972): Seasonal variation in the composition of the volatile oil of the leaves, buds, twigs of white spruce (Picea glauca). *Can. J. Bot.* **50**: 1595–1603.
— (1975a): Volatile leaf oil analysis in chemosystematic studies of North American conifers. *Biochemical Systematics and Ecology* **2**: 131–167.
— (1975b): Seasonal variation of the terpenes of the leaves, buds and twigs of blue spruce (Picea pungens). *Can. J. Bot.* **53**: 2978–2982.
— (1975c): Seasonal variation in the terpenes of the foliage of black spruce. *Phytochemistry* **14**: 1695–1700.
— (1978): Variation in leaf oil terpene composition of Sitka spruce. *Phytochemistry* **17**: 127–130.
— & M. J. HOLST (1968): Chemosystematic studies in the genus Picea (Pinaceae). III. The leaf oil of a Picea glauca × mariana hybrid (Rosendahl spruce). *Can. J. Bot.* **46**: 1–4.
SCHUCK, H. J. (1976): Quantitative Variation der Wachsauflage und der cuticulären Kohlenwasserstoffe bei Picea abies-Nadeln. *Flora* **165**: 303–314.
SCHÜTT, P. & H. J. SCHUCK (1973): Jahreszeitliche Schwankungen in der Zusammensetzung der Cuticularwachse von Pinus sylvestris. *Flora* **162**: 206–214.
SMELYANETS, V. P. (1977): Mechanisms of plant resistance in pine trees, Pinus sylvestris I. Indicators of physiological state in interacting plant-insect populations. *Z. Angew. Entom.* **83**: 226–233.
TJIA, B. & D. B. HOUSTON (1975): Phenolic constituents of Norway spruce resistant or susceptible to the eastern spruce gall aphid. *Forest Sci.* **22**: 180–184.
TJUKAVKINA, N. A., S. A. MEDVEDEVA & S. Z. IVANOVA (1975): Phenolics from needles of Larix dahurica Turcz. *Khimia drevjesni (Riga)*: 93–95.
VARAKSINA, T. N. (1973): Content of flavonoid glycosides in the needle of the Siberian larch. *Metabol. Khvoinykh Svyazi Period. Ikh. Rosta:* 40–48. = CA 082–05-28516Q.
WELLENDORF, H., U. KAUFMANN & M. HANSEN (1971): Thin-layer chromatography of fluorescent phenolic compounds in needles. A contribution to chemotaxonomy in Picea.

Forest Tree Improvement **2**: 19–39.

ZAFRA, M. & E. GARCIA-PEREGRIN (1976): Seasonal variation in the composition of Pinus halepensis and Pinus sylvestris twigs and needles essential oil. *J. agric. Sci. Camb.* **86**: 1–6.