Monoterpenes in the Volatile Leaf Oil of *Abies* × *arnoldiana* Nitz.

Arthur Koedam,* Johannes J. C. Scheffer, and Anders Baerheim Svendsen

The composition of the volatile oil from *Abies* × *arnoldiana* Nitz., a spontaneous hybrid of *A. koreana* Wils. and *A. veitchii* Lindl., has been investigated. The oil was analyzed by means of fractionation on silica gel, followed by gas chromatography over poly(*m*-phenyl ether) (six ring). Fifteen monoterpene hydrocarbons, three sesquiterpene hydrocarbons, *trans*-2-hexenal, and 16 oxygenated monoterpenes were identified. Isolations of the oil by the techniques of hydrodistillation and solvent extraction were compared. As a result it must be concluded that solvent extraction gives a more reliable picture of the terpene pattern present in the plant than distillation, as detrimental factors such as pH of distillation water, hydrolysis of labile components, and crystallization in the condenser of the distillation apparatus are ruled out. From this investigation it is also likely that santene and 5,6-dimethyl-5-norbornen-*exo*-2-ol are artifacts of distillation.

The present investigation was undertaken as part of a comparative study on the isolation procedures for essential oils. Previously we described the influence of distillation on the composition of the volatile oils from several umbelliferous fruits (Koedam et al., 1979a,b). In continuation of this series it was decided to investigate the volatile leaf oils of some species of the Coniferae to see if they also were subjected to changes in composition during isolation.

This paper is concerned with *Abies* × *arnoldiana* Nitz. (Pinaceae). This interesting tree was first raised in 1953 in the Botanical Garden of Göteborg (Sweden) from seed taken from a Korean fir (*Abies koreana* Wils.) at the Arnold Arboretum (United States). It was recognized as a natural hybrid of *Abies koreana* and *Abies veitchii* Lindl. by Nitzelius who gave also a botanical description (Nitzelius, 1970). It is a rather fast growing tree with a narrowly pyramidal habit. Leaf arrangement on the branchlets is reminiscent of *A. veitchii*, but the leaves (20–30 mm long) are broadened (2.5 mm) toward the truncate apex as in *A. koreana*. It bears its bluish-purple cones richly, even as a young plant (cf. *A. koreana*), and is increasingly popular as an ornamental tree. The species has also been created in Denmark by a controlled crossing in 1956 (Poulsen, 1979). MacGillivray (1960) stated that preliminary tests for breeding were carried out with several exotic *Abies* species, inter alia, *A. koreana* × *A. veitchii*, without indicating the origin. Finally, in a paper on interspecific hybridization between *Abies* species Klaehn and Winieski (1962) made mention of the artificial cross, giving Denmark (?1952) as the source.

However, so far no reports on the chemistry of the volatile leaf oil of this hybrid have been published. This study consists of two parts. First, an analysis is given of the monoterpenes in the volatile leaf oil of *A.* × *arnoldiana* and, second, experiments are described to evaluate the influence of distillation on the composition of the oil.

EXPERIMENTAL SECTION

Material. In January 1979, terminal branches of *A.* × *arnoldiana* were collected at random from a tree located near Lammenschans, Leiden (The Netherlands). The leaves (2.5 kg) were immediately separated from the twigs, thoroughly mixed (to provide average representative samples), and stored in plastic bags at −15 °C until use (within a few weeks).

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Isolation of the Oil. Initially the volatile oil was recovered by continuous distillation by using the apparatus described in the "European Pharmacopoeia" (1975). Five hundred milliliters of deionized water was added to a 200-g sample of leaves. Distillation at a rate of 2.5 mL/min during 6 h afforded 1.55 mL of oil (0.78%, v/w). The oil obtained in this manner was used for identification of the components by gas chromatography. All further distillations were carried out with the same apparatus, but with 20-g leaf samples. The influence of the pH of the distillation water was determined by distilling the plant material for 4 h in 300 mL of buffer solutions (McIlvaine buffers, pH range 2.2–8). Some distillations were performed over a 16-h period to check if the length of the distillation caused the oil composition to change. Every hour a sample of 0.5 μL was taken and diluted with 0.25 mL of redistilled pentane before gas chromatography.

The sequence of recovery of the different compounds was examined by using a Likens and Nickerson apparatus of the type described by Maarse and Kepner (1970). The sample side contained 20 g of leaves in 300 mL of deionized water and the solvent side contained 10 mL of redistilled pentane. Total extraction time was 8 h. To obtain separate fractions of the distillate, we replaced the vessel with the organic solvent after every 30 min.

Solvent extracts were prepared for comparison: 10 g of leaf material was ground with solid carbon dioxide and extracted with a refluxing pentane/diethyl ether mixture (1:1) during 6 h, and the extract was concentrated to 5 mL as described elsewhere (Koedam et al., 1979a).

Column Chromatography. The oil obtained with the apparatus of the "European Pharmacopoeia" was fractionated over silica gel (40 g, Merck), using a glass tube (18 mm i.d.) with a cooling jacket kept at 10 °C. The adsorbent was poured into the column as a pentane slurry. For *monoterpene hydrocarbons* we chose a mixture of 75% 70–230 mesh silica gel 60 and 25% 230–400 mesh silica gel 60. Sample size was 0.5 mL. The column was eluted with pentane. The eluate was collected in small fractions as reported previously (Scheffer et al., 1976a). *Oxygenated compounds* were chromatographed on silica gel 60, 70–230 mesh. Elution of the oil (0.75 mL) was carried out by applying a 2.5–50% gradient of diethyl ether in pentane and collecting a number of fractions (Scheffer et al., 1977). In both cases the fractions were concentrated to a volume of 1 mL under reduced pressure at 0 °C. Rearrangements during chromatography were prevented by adding 5% water to the adsorbent (Scheffer et al., 1976b).

Gas Chromatography (GC). Analyses were carried out on a Packard GC Model 409 (Packard-Becker B.V., Delft,

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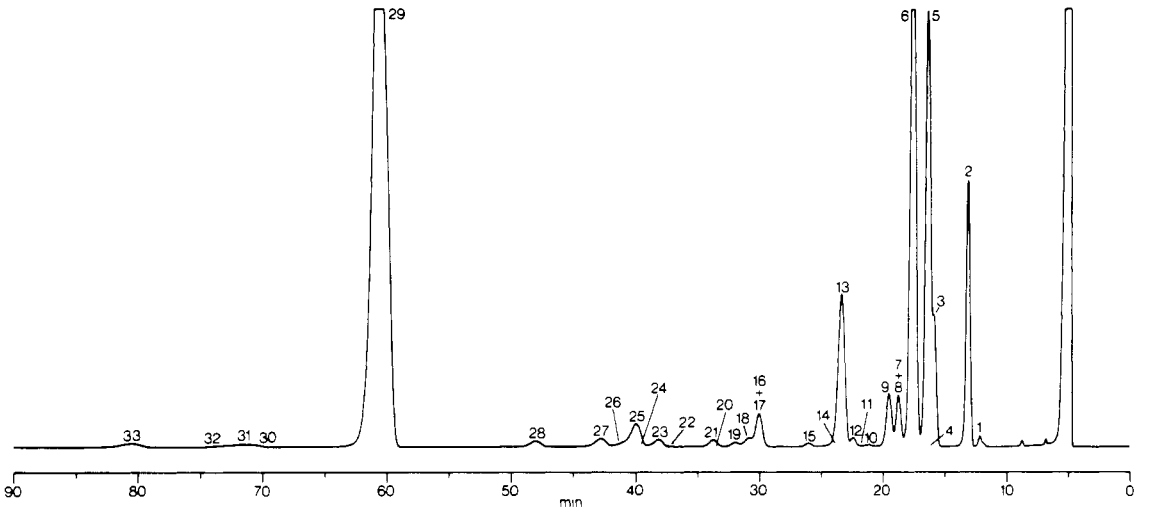

Figure 1. Gas chromatogram showing volatile leaf oil composition of *A.* × *arnoldiana* after 6 h of distillation. Column: poly(*m*-phenyl ether) (six ring) at 140 °C. Peak numbering as in Table I.

The Netherlands) equipped with a FID. Conditions employed were as follows: copper column, 8 m × 1.5 mm i.d.; stationary phase, 10% poly(*m*-phenyl ether) (six ring) on Chromosorb W AW (60–80 mesh); injector and detector temperatures, 200 °C; oven temperature, 80 °C (monoterpene hydrocarbons) and 140 °C (oxygenated compounds); the N_2 carrier flow was maintained at 18 mL/min (80 °C) and 14 mL/min (140 °C); sample size, 1 μL. Peak areas were measured with an Infotronics CRS-208 integrator (Infotronics Corporation Ltd., Shannon, Ireland).

Identification of Components. This was assigned by comparison of retention data with those of authentic samples on columns of different polarity (Carbowax 20M, β,β′-oxydipropionitrile and SF-96). Where this procedure failed to yield conclusive identification, compounds were isolated by means of preparative GC from the fractions obtained with column chromatography. Subsequently mass spectra were recorded. Details were published elsewhere (Koedam and Gijbels, 1978; Scheffer et al., 1978).

RESULTS AND DISCUSSION

Oil Composition. As a stationary phase for GC separation of the oil components we chose poly(*m*-phenyl ether) (six ring). This liquid was recommended by Tyson (1975) for GC–MS analysis of monoterpene hydrocarbons. According to our experience it is equally well suited for GC of oxygenated monoterpenes. It also has the advantage that the sesquiterpenes are eluted *after* all the monoterpenes. Thus these two groups do not interfere as is the case on the generally used Carbowax 20M columns.

Figure 1 shows a gas chromatogram of the volatile leaf oil of *A.* × *arnoldiana* after a distillation period of 6 h, using the apparatus described in the "European Pharmacopoeia". A list of compounds indentified in the oil as well as their percentages is given in Table I. As will be seen bornyl acetate is the predominant compound of the oil (38.3%), while camphene (24.8%), α-pinene (16.3%), santene (6.2%), and limonene (5.5%) are the major monoterpene hydrocarbons.

An overlap of several compounds within the range of monoterpenes is usually observed during GC. This can be alleviated by means of column chromatographic prefractionation on silica gel (Scheffer et al., 1976a, 1977). Thus, a GC run at 80 °C initially revealed 12 compounds of the monoterpene hydrocarbon fraction. The oxygenated fraction (at 140 °C) contained another 13 substances. The enrichment of the compounds in the various fractions obtained after column chromatography facilitated GC and

Table I. Percentages of Compounds Found in the Volatile Leaf Oil of *A.* × *arnoldiana* after Distillation during 6 h

no.	compound	%
1	*trans*-2-hexenal[a,b]	0.1
2	santene[a]	6.2
3	tricyclene[a]	2.0
4	α-thujene[a]	Tr[d]
5	α-pinene[a]	16.3
6	camphene[a]	24.8
7	sabinene[a]	Tr
8	myrcene[a]	1.1
9	β-pinene[a]	1.2
10	α-phellandrene[a]	0.1
11	Δ_3-carene[a]	Tr
12	α-terpinene[a]	0.2
13	limonene[a]	5.5
14	β-phellandrene[a]	0.5
15	γ-terpinene[a]	0.1
16	terpinolene[a]	0.8
17	linalool[a,b]	Tr
18	5,6-dimethyl-5-norbornen-*exo*-2-ol[a,b]	0.1
19	unknown (M^+ *m/e* 152)	0.1
20	α-fenchol[a,b]	Tr
21	campholenic aldehyde[a,b]	0.2
22	camphor[a,b]	Tr
23	camphene hydrate[c]	0.2
24	isoborneol[a,b]	0.1
25	borneol[a,b]	0.9
26	terpinen-4-ol[a,b]	0.2
27	α-terpineol[a,b]	0.3
28	fenchyl acetate[a,b]	0.2
29	bornyl acetate[a,b]	38.3
30	citronellyl acetate[a,b]	Tr
31	α-terpinyl acetate[a,b]	0.1
32	neryl acetate[a,b]	Tr
33	geranyl acetate[a,b]	0.1

[a] Identity confirmed by comparison of GC retention data with those of authentic samples on columns of different polarity. [b] Mass spectrum consistent with that of authentic sample. [c] No reference substance available, but mass spectrum identical with that published by Stenhagen et al. (1974). [d] Tr = trace (area less than 0.1%).

MS identification of a number of trace constituents. In this manner the presence of α-thujene, sabinene, and Δ_3-carene in the oil was established. Likewise, in the oxygenated portion of the oil several compounds were found which could hardly be seen on the chromatogram of the total oil. Trace amounts of camphor, citronellyl acetate, and neryl acetate were thus determined. Furthermore, due to the separation obtained by column chromatography it

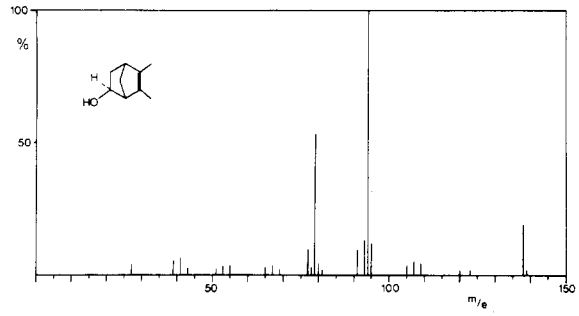

Figure 2. Mass spectrum of 5,6-dimethyl-5-norbornen-*exo*-2-ol.

became evident that linalool was present in trace amounts under the monoterpene hydrocarbon terpinolene. The difference in elution from the silica column also accounts for the detection of α-fenchol (hidden under campholenic aldehyde) and of isoborneol (obscured by borneol). In addition, traces of some other substances with GC retention times between those of terpinolene and camphene hydrate were observed in several fractions. Mass spectra of these unknowns showed M^+ values of m/e 140 and 154, but further identification was unsuccessful. However, as the elution sequence during column chromatography provides extra information on the functional group of a compound (Scheffer et al., 1977), it is highly probable that these substances are (monoterpene) alcohols. With regard to compound 19, it should be noted that its mass spectrum was identical with that of an authentical sample of α-pinene oxide, but the GC retention time on columns of different polarity varied slightly. The presence of 5,6-dimethyl-5-norbornen-*exo*-2-ol in the oil is remarkable. To our knowledge this compound has thus far only been identified by Demole et al. (1976) in the oil of East Indian sandalwood (*Santalum album* L). Its mass spectrum is presented in Figure 2.

A GC run at 180 °C revealed that some sesquiterpene hydrocarbons were also present in the oil, of which caryophyllene, β-elemene, and α-humulene could be identified. Lack of reference substances prevented unequivocal identification of other compounds in this class and they were not taken into consideration during further study.

Interesting to note is the marked difference observed in the oil composition when several samples of the plant material were submitted to distillation after a 3-week storage period at −15 °C. The bornyl acetate content of this oil was low (about 23%), but a considerable increase, up to 13%, in the amount of borneol had taken place. As all material was collected at one time during the dormant period, seasonal variation can be excluded (Adams, 1979). Probably certain cell structures are ruptured during storage in frozen condition, thus permitting interaction between bornyl acetate and acidic cell contents. The background of this hydrolysis has to be the subject of future work.

Influence of Distillation Period. First it was investigated if the ratio of hydrocarbons vs. oxygenated compounds changed during the distillation of the volatile oil from the plant material, as was previously found for umbelliferous seeds (Koedam et al., 1979a,b). GC analysis of the oil samples taken every hour (European Pharmacopoeia apparatus) revealed that the ratio of both groups varied only during the first hours of distillation. After 1 h the proportion of hydrocarbons to oxygenated compounds was 54:46; after 2 h the ratio was 60:40. Upon further distillation practically no changes could be observed and the ratio of the two groups remained nearly constant.

Subsequently, an experiment was carried out with the Likens–Nickerson apparatus. Sequential oil fractions were

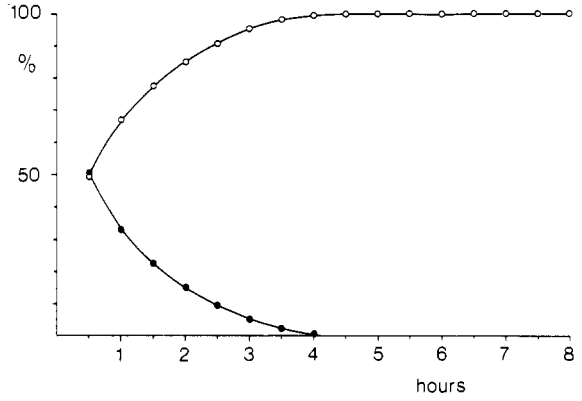

Figure 3. Relative proportions of hydrocarbons (○) and oxygenated compounds (●) in sequential fractions of *A.* × *arnoldiana* oil, sampled separately during 0.5-h intervals.

obtained by replacing the pentane flask. Again the ratio of hydrocarbons vs. oxygenated compounds was determined. Results are given in the graph of Figure 3. The first fraction (30 min) contains approximately equal amounts of both hydrocarbons and oxygenated compounds. With continuing distillation, the portion of oxygenated compounds in the fractions declines quickly with an attendant increase in the hydrocarbons. In the fraction collected between 3.5 and 4 h only traces of bornyl acetate were found. Thereafter no oxygenated compounds were detected in the distillate. As Figure 3 shows, further fractions were only made up of hydrocarbons, though in very small quantity. It must therefore be concluded that the higher boiling oxygenated compounds distill before the hydrocarbons. This is in agreement with our previous findings (Koedam et al., 1979a,b), and the conclusion may be drawn that also in the case of *A.* × *arnoldiana* the diffusion of the oil components out of the cell structures is of greater importance than the boiling points of the compounds in question. As the oxygen-containing compounds are much more soluble in the hot distillation water than the hydrocarbons, diffusion of the first is highly favored. This phenomenon, known as hydrodiffusion, has been described in detail by Von Rechenberg (1910).

That the composition of the total oil is only slightly affected by the difference in distillation rate of both fractions is due to the greater part of the oil distilling within the first 2 h. The later fractions, although practically exclusively composed of hydrocarbons, are of minor importance from a quantitative point of view.

It was also observed that the relative amount of santene rapidly increased in the later fractions. For instance, the fraction distilling between 0.5 and 1 h contained 6.5% of this compound, whereas in the fraction collected between 6.5 and 7 h santene was the largest compound with 78.8%. As it is not likely that santene is much less soluble than the other monoterpene hydrocarbons in the boiling distillation water, and therefore more strongly retained by the plant material, we are probably dealing with an artifact.

Influence of pH. In an investigation of the volatile leaf oil of *Pinus pinaster* Ait., Pauly et al. (1973) reported that the hot distillation liquid rapidly showed an acidic pH (around 3). These authors suggested that this caused hydrolysis of thermally labile terpene alcohol esters such as linalyl and α-terpinyl acetate since the alcohols of both compounds were present in distilled oils but absent from oils obtained by maceration of the leaves in organic solvents. As the oil under investigation in the present study was rich in bornyl acetate, we also examined the influence of the pH of the distillation water on the composition of the oil. For this purpose the plant material was distilled

Table II. Percentages of Borneol and Bornyl Acetate in the Volatile Fraction of *A.* × *arnoldiana* Leaves Soaked in Solutions of Various pH's for 16 h at Room Temperature

	pH			solvent extract
	2.2	5	7	
borneol	16.5	11.7	4.2	0.1
bornyl acetate	18.0	24.1	31.4	36.0

from buffered mediums of various pH's between 2.2 and 8. In view of the results presented in Figure 3 distillations were conducted for 4 h. Compared to the amount of borneol in solvent extracts (0.1%) the borneol content increased at a pH between 5 and 8 to about 1.0% and was somewhat higher at low pH (≤4). Apparently, bornyl acetate is only slightly affected by the acidity of the distillation water. However, when coarsely chopped leaf material was placed in buffered mediums at various pH's (16 h, room temperature) and subsequently extracted with a pentane/diethyl ether mixture (1:1), the data indicated in Table II were obtained. From these figures it may be concluded that acid-catalyzed hydrolysis of bornyl acetate takes place. Kinetics of this reaction have been discussed by Bunton et al. (1965) and by Lajunen (1974). Obviously this hydrolysis only takes place to a small extent during distillation. This seems rather surprising, especially in view of the high temperature. It should, however, be remembered that on the basis of hydrodiffusion (vide supra) the oxygenated compounds are available for distillation much faster than the hydrocarbons. Thus, bornyl acetate is in contact with the acidic medium for a relatively short period (in fact, limited to the first hour of distillation), whereby extreme hydrolysis of this compound is prevented.

Furthermore, in the experiment on the pH influence of the distillation water, a striking difference was observed in the amount of santene. At pH 2.2 the content of santene in the oil was 2.2% but it increased gradually with the pH value of the distillation medium to more than 8% at pH 8. The inverse was observed with 5,6-dimethyl-5-norbornen-*exo*-2-ol: 0.5% at pH 2.2, decreasing to traces at pH values 6–8. When, in comparison, solvent extracts from ground material were investigated, only a small quantity of santene (0.2%) was present. Likewise, no 5,6-dimethyl-5-norbornen-*exo*-2-ol could be detected. These compounds are apparently artifacts generated during distillation and influenced by the pH of the distillation water, but so far the mode of formation is not understood. In this point our findings are in contrast with those by Von Rudloff and Hunt (1977), who did not observe any differences between distilled oils and the volatile portions of solvent extracts from *Abies amabilis* (Dougl.) Forbes. With regard to the presence of santene it is also of interest to note that Von Rudloff (1975) reported reasonable amounts (1–5%) of this compound in some fir species but only traces in others. The author assumes the presence (or absence) of a specific enzyme for the formation of santene from camphene. However, in view of our results, the influence of different isolation conditions must not be overlooked. This aspect warrants further research.

Apart from the influence of the pH of the water, there is another disadvantage of distillation worth mentioning. Initially, plant material that was soaked in buffer solutions at room temperature (to see if hydrolysis of bornyl acetate would occur) was submitted to distillation instead of solvent extraction. Contrary to expectation the distillates contained almost no borneol. However, inspection of the distillation apparatus revealed a thin film of crystals in the upper part of the condenser, which proved to be borneol. This problem was also encountered by other authors with camphor (Von Rudloff, 1968; Lawrence et al., 1970). The solution of Joye et al. (1971) whereby "product temperature was maintained at 40–50 °C to prevent crystallization of high-melting components such as borneol" seems undesirable in view of the risk of volatilization of the lower boiling monoterpenes. For the same reason, the instruction found in the literature (Moritz, 1938; Koch, 1939; Flück et al., 1949; Kaiser and Lang, 1951) to "rinse" the condenser by cutting off the water supply toward the end of the distillation is highly unfavorable, although Bauermeister and Hagenström (1955) claim the opposite. Moreover, with this procedure the temperature of the distillate rises quickly. As the water solubility of the oil is greatly increased at higher temperatures (Cocking and Middleton, 1935; Petersen, 1952; Schirm, 1953), part of the oil will be dissolved in the recirculating aqueous phase. A new source of losses is then introduced. To overcome the inconvenience of crystallization and to prevent volatilization and enhanced dissolution, as outlined above, in our experiments we washed the condenser apparatus, after removal of oil and water, with small amounts of an organic solvent (e.g., pentane). This permits at the same time the washing down of and the collection of oil traces adhering to the surface of the graduated tube. This procedure will, of course, affect the physical properties of the oil, but, what is more important, it does not interfere with GC analysis.

Finally, it should be emphasized that these results are based on laboratory-scale experiments. The observed distillation effects are more pronounced with larger batches of plant material since the longer distillation time required to exhaust the material is obviously connected with longer residence time of the volatile constituents under unfavorable conditions.

ACKNOWLEDGMENT

Appreciation is expressed to Tor G. Nitzelius, Våxtorp (Sweden), for the identification of plant material, to Erich Ziegler, Aromachemie, Aufsess (G.F.R.), and to F. J. Hammerschmidt, Dragoco, Holzminden (G.F.R.), for several reference substances, to Edouard Demole, Firmenich S. A., Geneva (Switzerland), for an authentic sample of 5,6-dimethyl-5-norbornen-*exo*-2-ol as well as for the provision of some mass spectra, and to Anja Looman for technical assistance.

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Received for review November 13, 1979. Accepted March 18, 1980. This paper is Part 3 in the series "Comparison of Isolation Procedures for Essential Oils". For Part 2, see Koedam et al. (1979b). Preliminary report presented at the "10. Internationale Arbeitstagung Vorkommen und Analytik ätherischer Öle", Würzburg, G.F.R., May 1979.

Some Effects of Nitrogen Fertilizer on the Chemical Composition of Pearl Millet Grain

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Five pearl millet [*Pennisetum americanum* (L.) Leake] hybrids grown at two fertilizer levels during crop years 1976 and 1977 at Tifton, GA, were studied to determine the effect of nitrogen fertilizer on the millet grain. The millets studied were inbred hybrids Tift 18DB, Tift 23DB, and Tift 383 and the F_1 hybrids of Tift 23DA × Tift 18DB and of Tift 23DA × Tift 383. Nitrogen fertilizer levels were 12 lb/acre and 120 lb/acre. Protein content was increased by 19 to 55% in these hybrids by the increased use of fertilizer, with only minimal effect on the quality of the protein. Protein content varied from 8.8 to 14.1% for the hybrids grown at the low level of fertilizer and from 11.6 to 20.5% for those grown at the high level. Starch content was inversely related with protein content. Crude fiber showed little variance with fertilizer level. Mineral content was variable in the samples.

Pearl millet is a major world food grain, the staple of many Africans and Asians. It has better protein quality than most other cereals and a protein content ranging from 8 to 20.9% (Burton et al., 1972; Bailey et al., 1979). Pearl millet also contains significant quantities of the minerals necessary for good nutrition in people and animals (Casey and Lorenz, 1977). Comparative studies of protein quality and mineral constituents of some Indian varieties have shown that the high-protein varieties contain plentiful amounts of essential amino acids and calcium, phosphorus, and potassium. The influence of nitrogen fertilizer levels on some Indian varieties has also been investigated with respect to protein quality (Deosthale et al., 1972) and iron and magnesium uptake (Skukla and Bhatia, 1971). In the present report, the effect of nitrogen fertilizer was observed on the protein and starch content, amino acid composition, and the mineral uptake of five dwarf pearl millet inbreds and hybrids over two crop years.

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MATERIALS AND METHODS

Grain samples of five pearl millet inbreds and hybrids grown in the 1976 and 1977 seasons were obtained from the Georgia Coastal Plain Experiment Station, Tifton, GA. The millets were inbreds Tift 18DB, Tift 23DB, and Tift 383 and the F_1 hybrids of Tift 23DA × Tift 18DB and of Tift 23DA × Tift 383. They were grown on moderately fertile loamy sand at two levels of nitrogen fertilizer (12 lb/acre and 120 lb/acre), on two replicate plots. The base fertilizer (12 lb of N/acre) was 5–10–15 applied to all the plots with the additional nitrogen applied in the form of ammonium nitrate.

Clean pearl millet grain, free of glumes and broken kernels, was evaluated as received, without being dried, dehulled, or milled. Lipid-free meal was obtained by homogenizing and extracting the whole grain with petroleum ether in a laboratory tissue homogenizer and separating the supernatant and flour by centrifugation (3000*g* for 10 min). The flour was air-dried and ground with a mortar and pestle until it passed through a 60-mesh screen. The protein content of the lipid-free whole meal was estimated (dry weight basis) by the macro-Kjeldahl procedure (N × 6.25). The starch content was determined by polarimetric method 76-20 (AACC, 1971) on the lipid-free meal. The mineral content was determined on the whole millet pressed at 20 000 lb/in. for 10–15 s in a Sonar cap