Physiological modulation of the vitality of Scots pine trees by atmospheric ammonia deposition

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7. Nitrogen metabolism in forest stands under conditions of relatively high atmospheric nitrogen deposition [7]

7.1. Abstract

Nitrogen metabolism of the needles of forty-year-old Douglas fir and Scots pine trees, growing in two forest stands on cation-poor and acidic sandy soil with a relatively high atmospheric nitrogen deposition was studied. The composition of the free amino acid (FAA) pool, the concentrations of total nitrogen and soluble protein, and the activities of glutamine synthetase (GS) and glutamate dehydrogenase (GDH) were determined in the needles. An excessive nitrogen supply by a high atmospheric nitrogen deposition in both forest stands was indicated by the high concentrations of total nitrogen and the amino acids arginine, glutamic acid, glutamine and aspartic acid in control trees. In addition, the effect of optimal nutrition and water supply (fertigation) on the needle nitrogen metabolism was evaluated. The total concentration of the FAA pool in needles of both tree species was lower in the fertigated than in the non-fertigated (control) trees, except for one-year-old needles of Scots pine, in which the concentration after fertigation did not differ from the control. The lower total FAA concentration in the fertigated trees could be attributed to arginine, which concentration was on average 60 % lower than in the control. Neither the concentration of soluble protein nor the activity of GS were influenced by fertigation. The activity of GDH in fertigated trees only differed significantly from the control in October. Scots pine needles had higher concentrations of protein (50 %) and higher activities of GS (44 %) and GDH (25 %) than Douglas fir needles. Possible explanations for the lower vitality of Douglas fir compared to Scots pine are given.

7.2. Introduction

The increase in deposition of atmospheric nitrogen (N) observed in Europe (Heij & Schneider, 1991), has dramatic effects on forests ecosystems (Nihlgård, 1985; Heinsdorf, 1991). Increases in N concentration of the leaves to levels far above the 1.8 %, considered optimal for production (Von Heinsdorf and Krauß, 1991), may cause an enlargement of the nutrient imbalance (Van Dijk and Roelofs, 1988), a decrease in root/shoot ratio (Van Dijk et al., 1990), a decline in mycorrhiza association (Termorshuizen, 1993), and

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Table 7.1. Nutrient additions (kg N ha^{-1} y^{-1}) to the fertigation plots (De Visser et al. 1994).

	N	P	K	Mg	Ca
Douglas fir	39	36	60	4.8	6.0
Scots pine	31	13	65	7.0	8.5

Table 7.2. Water additions (mm) to the fertigation plots (De Visser et al. 1994).

	year	total	period
Douglas fir	1990	263	11-04/22-10
	1991	277	7-05/7-10
Scots pine	1990	304	20-05/25-09
	1991	240	7-05/27-09

an increased sensitivity to frost (Dueck et al., 1991), to drought (Van der Eerden et al., 1991) and to diseases (Flückiger & Braun, 1992). The situation is particularly complex in coniferous forests, which have been planted on soils with a low availability of N (Van Breemen & Van Dijk, 1988). Two important conifer species in Dutch forests, Douglas fir and Scots pine, show large differences in vitality; the percentage of vital trees in 1992 was 9.1 for Douglas fir and 62.1 for Scots pine (Smits, 1992). In addition, the vitality of Douglas fir steadily decreased, in 1992 being only half of the percentage in 1988, while in Scots pine it has remained stable since 1988.

NH_y (NH_3+NH_4^+) is the main component of atmospheric N deposition in forests (Erisman, 1991). NH_3 is largely absorbed by the shoots, while NH_4^+ uptake mainly occurs via the roots. Gaseous NH_3 is directly absorbed through the stomata (Van Hove et al., 1992) and dissolved in the leaf to NH_4^+. Glutamine synthetase (GS) and glutamate synthase (GOGAT) are responsible for 95 % of the assimilation of NH_4^+ in the shoots and roots of higher plants (Lea et al., 1992). Nitrate taken up by the roots is also reduced to NH_4^+ and incorporated in the organic N pool in the same way (Oaks, 1986). From the resulting pool, the accumulation of specific free amino acids (FAA) like arginine, has been directly related to the N status of conifers (Van Dijk & Roelofs, 1988; Näsholm & Ericsson, 1990).

Investigation of the N metabolism in needles of Scots pine seedlings, fumigated with NH_3 in controlled-environment chambers, showed increased levels of GS activity and increased concentrations of soluble protein and the FAA arginine, glutamic acid, aspartic acid and glutamine by NH_3 fumigation (Pérez-Soba et al., 1994a). Hence, changes in these parameters indicate an adaptation of Scots pine to high atmospheric NH_3 concentrations. The purpose of this study was to test whether the results obtained with seedlings under artificial conditions in the laboratory also applied to mature trees under field conditions with a relatively high atmospheric N deposition. In addition, we studied the effect of optimalization of the nutrient and water supply (fertigation) on N metabolism of the

mature trees in the cation-poor and acidic soil of the forest stand. The optimalization was performed in order to reach a balanced nutrition in relation to the N input in the soil and an optimal water supply for tree growth (De Visser, 1990). Comparison of the results might give evidence for differences in vitality of Scots pine and Douglas fir as observed in Dutch forests.

7.3. Materials and methods

Plant material, soil type and treatments

In this study two stands, one of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) and one of Scots pine (Pinus sylvestris L.) were investigated. Both forests are located in the central area of The Netherlands called "De Veluwe". The Douglas fir stand was planted as 2-year-old seedlings in coversand in 1953 and in the Scots pine stand seeds were sown in a cation-poor, acidic sandy soil in 1952. The chemical properties of the A_p horizon (0-20 cm) of Douglas fir were: pH (H_2O) 3.8; pH (1M KCl) 3.1; C (%) 3.1; N (%) 0.11; CEC (0.01 M $BaCl_2$) 2.8 $cmol_c\ kg^{-1}$ soil; exchangeable cations (0.01 M $BaCl_2$): 0.10 Na, 0.20 Ca, 0.03 Mg, 0.06 K $cmol_c\ kg^{-1}$ soil. The chemical properties of the A_h horizon (0-20 cm) of Scots pine were: pH (H_2O) 4.3; pH (1M KCl) 3.3; C (%) 1.6; N (%) 0.07; CEC (0.01 M $BaCl_2$) 2.9 $cmol_c\ kg^{-1}$ soil; exchangeable cations (0.01 M $BaCl_2$): 0.15 Na, 0.43 Ca, 0.11 Mg, 0.10 K $cmol_c\ kg^{-1}$ soil. The atmospheric N deposition in both forest stands was ca. 40 kg N $ha^{-1}\ y^{-1}$. A detailed description of soil conditions, precipitation, throughfall and further details is given by De Visser (1990). Two sample areas in each stand were used: a control, exposed to ambient air pollution, and an irrigation-fertilization treatment (hereafter referred to as fertigation). Fertigation started in July 1988 for the Scots pine stand and in May 1989 for the Douglas fir stand. A complete nutrient solution, containing macronutrients (N, P, K, Mg and Ca) and micronutrients (Fe, Mn, B, Mo, Zn and Cu), was injected into the irrigation water (for composition see Tables 7.1 and 7.2). Nutrients were supplied in optimal proportion to the N requirement of the trees (Ingestad, 1988). Annual uptake was estimated to be 120 and 100 kg N $ha^{-1}\ y^{-1}$ for Douglas fir and Scots pine, respectively (De Visser, 1990). Nitrogen was applied as NH_4NO_3.

We selected trees with average height and basal area (for pine and fir, 10 and 17 m and 22 and 28 $m^2\ ha^{-1}$, respectively; standard deviations < 5%), 6 trees per stand (3 trees per treatment) for enzyme and protein determination and 20 (10 trees per treatment) for amino acid investigation. Enzyme activities and protein concentrations were determined 5 times during a two-year period. Sampling of Douglas fir took place on February 15, May 23 and October 16, 1990 and on June 26 and October 15, 1991. Sampling of Scots pine took place on February 2, May 21

and October 10, 1990 and on June 6 and October 25, 1991. The needles harvested in February, June and October 1990 were formed in 1989 and those harvested in May and October 1991 were formed in 1990. The FAA pool was studied in one-year-old and in current-year needles once, in August 1989. Analyses were always performed on needle material from the same branch, located at the sunny side of the seventh and fourth whorl of Douglas fir and Scots pine, respectively.

Extraction and determination of various parameters in the needles

Needle extract for soluble protein and enzyme determinations

Needles were harvested between 10:00 and 10:30 h to minimize diurnal variations found in previous experiments (Pérez-Soba et al., 1994a). They were immediately frozen in liquid nitrogen on site and kept there during transport to the laboratory. Needles were extracted two hours later to determine enzyme activities and soluble protein concentrations according to Pérez-Soba et al. (1994a). Three parallel determinations were carried out on each tree. Preliminary experiments showed that GS activities of needles, kept for two hours in liquid nitrogen, were about 5% lower than activities of needles extracted immediately after harvest.

Soluble proteins

The concentration of soluble proteins was determined as described by Bradford (1976), using the BIO-RAD reagent and a BSA (bovine serum albumin) standard. Samples were diluted 1:100 and a microassay was performed as reported by Pérez-Soba et al. (1994a).

Glutamine synthetase and glutamate dehydrogenase

GS (EC 6.3.1.2) activity was determined with the hydroxamate synthetase assay (O'Neal & Joy, 1973) as described by Pérez-Soba et al. (1994a) for Scots pine needles. Trial experiments showed the incubation media to be also suitable for Douglas fir. The aminating activity of GDH (EC 1.4.1.2) was determined according to Pahlich & Joy (1971). Enzyme activities are given as $\mu mol\ h^{-1}\ g^{-1}$ fresh weight and as $\mu mol\ h^{-1}\ mg^{-1}$ protein (specific activity).

Free amino acid pool (FAA)

Needles were homogenized in liquid nitrogen and water-soluble amino acids were extracted according to MacKenzie & Holme (1984), with a mixture of 37 % chloroform, 48% methanol and 15% water. Fifteen ml of the extraction solution were added to 400 mg of freeze-dried needle sample three times. Each time the mixture was shaken for 2 min and centrifugated at 36 200 g for 15 min. The 3 supernatants were combined, mixed and split into 3 portions of 15 ml to which 4.7 ml chloroform and 6 ml water were added. After shaking for 1 min and centrifugation for 15 min, the superficial aqueous layer was sampled, dried with a rotary film evaporator, dissolved in 10 ml 0.1 M HCl and stored at -20 ^{o}C. The

extraction solution was cleaned on an acid resin column (Dowex 50X8), whereby the adsorbed amino acids were rinsed off with 2 M NH_4OH solution, followed by addition of an internal standard, nor-leucine. The residue was stored at -20 ^{o}C. The amino acids were esterified with isobutanol, dissolved in acetyl chl o-ride and acylated with heptafluor butyric anhydride (MacKenzie, 1987). The N-heptafluorbutyryl isobutyl esters were

Figure 7.1. Soluble protein (mg g^{-1} FW) (a,b) and activities of GS (c,d) and GDH (e,f) (µmol h^{-1} g^{-1} FW) in needles of Douglas fir and Scots pine trees non-fertigated (open bars, control) or fertigated (shaded bars). Samples were harvested in February, May and October 1990 and in June and October 1991 and consisted of one-year-old needles. Significant differences between fertigation and control are indicated by * ($P<0.05$) and ** ($P<0.01$). Each bar represents the mean of 3 samples.

Table 7.3. Concentration of abundant free amino acids μmol g^{-1} DW) within the FAA pool and total concentration of the FAA pool, in needles of Douglas fir and Scots pine trees, harvested in August 1989. C, control; F, fertigated. Mean values of three parallel determinations in a mixed sample of ten trees. Contribution of each amino acid (%) to the total free amino acid pool is indicated between brackets.

		Douglas fir		Scots pine	
		C	F	C	F
Current-year	Arg	235.1 (53)	16.6 (16)	113.3 (37)	32.4 (15)
	Asp	29.7 (7)	21.7 (21)	27.9 (9)	39.2 (18)
	Glu	50.0 (11)	28.1 (27)	40.5 (13)	50.3 (23)
	Gln	28.4 (6)	0.8 (0)	17.5 (6)	7.7 (3)
	His	33.5 (8)	8.3 (8)	23.1 (7)	8.0 (4)
	Pro	10.6 (2)	4.8 (5)	39.9 (13)	26.2 (12)
	Total	439.8	103.5	309.6	221.2
One-year-old	Arg	80.2 (32)	33.9 (22)	144.7 (53)	110.2 (35)
	Asp	25.5 (10)	27.1 (18)	17.3 (6)	42.6 (13)
	Glu	52.7 (21)	43.9 (28)	23.9 (9)	12.0 (4)
	Gln	16.8 (7)	4.7 (3)	12.0 (4)	12.4 (4)
	His	16.3 (7)	8.1 (5)	10.0 (4)	5.3 (2)
	Pro	9.8 (4)	5.4 (4)	24.1 (9)	34.2 (11)
	Total	246.6	154.1	272.5	317.4

analyzed by gas chromatography (HP 5830).

Statistics

The concentration of soluble protein and the activities of GS and GDH were evaluated by analysis of var i-ance. We performed a square root transformation to stabilize the var i-ances of the GS and GDH activities. Following the analysis of variance we calculated the LSD for testing pairwise diffe rences.

7.4. Results

Comparison of optimal nutrient and water supply with control (cation-poor and acidic soil)

The concentration of soluble protein and the activity of GS in needles of

Table 7.4. Nitrogen concentrations (% DW) in one-year-old needles of Douglas fir and Scots pine trees. C, control; F, fertigated. Mean values ± SD. The number of samples is indicated between brackets.

	Douglas fir		Scots pine	
	C	F	C	F
October 1990	2.2 ± 0.5 (9)	2.1 ± 0.2 (7)	2.1 ± 0.1 (9)	1.8 ± 0.7 (7)
October 1991	2.1 ± 0.3 (10)	1.9 ± 0.2 (9)	1.7 ± 0.2 (11)	1.5 ± 0.1 (9)
October 1992	2.0 ± 0.3 (10)	1.6 ± 0.1 (4)	1.9 ± 0.1 (9)	1.5 ± 0.2 (10)

fertigated trees did not differ signif i-cantly from the controls (Fig. 7.1a, b, c and d). Only in February 1990, the concentration of soluble protein in fertigated Scots pine was signif i-cantly higher than in the control. The activity of GDH in Douglas fir ne e-dles was about 45% higher in the fertigated trees than in the controls in October 1990 and 1991 (Fig. 7.1e). The activity of GDH in Scots pine was significantly lower in the fertigated trees than in the controls: 8% in February 1990 and 20% in October 1990 and 1991 (Fig. 7.1f).

From the nineteen amino acids analyzed, arginine (Arg), aspartic acid (Asp), glutamic acid (Glu), glutamine (Gln), histidine (His) and proline (Pro) were the most abundant within the FAA pool in needles of control and fertigated Douglas fir and Scots pine trees (Table 7.3). Fertigation resulted in the following

Table 7.5. Soluble protein (mg g^{-1} FW) and specific activities of GS and GDH (mmol h^{-1} mg^{-1} protein) in one-year-old needles of Douglas fir and Scots pine harvested in 1990 and 1991. Data of the control and fertigation treatments were pooled within each tree species. Means of six samples. Significant differences between tree species are indicated by * ($P<0.05$), ** ($P<0.01$) and *** ($P<0.001$). ns, not significant.

	Tree species	February 90	May 90	October 90	June 91	October 91	Mean
Protein	Douglas fir	10.1	8.2	10.7	6.2	5.0	8.1
	Scots pine	12.1	12.6	13.5	9.0	12.7	11.98
GS	Douglas fir	7.0	2.0	0.6	4.7	4.9	3.8
	Scots pine	4.4	5.3	2.3	3.5	12.1	5.5
GDH	Douglas fir	6.1	3.9	2.5	4.4	6.5	4.7
	Scots pine	8.7	5.0	6.6	6.1	2.9	5.9
Protein		**	**	ns	***	***	***
GS		ns	***	**	ns	**	**
GDH		*	ns	***	*	*	*

changes: (1) a decrease of the total FAA pool concentration, mainly caused by a decrease in Arg concentration, which no longer was the highest within the pool, (2) an increase in the contribution of Asp and Glu to the FAA pool, except in one-year-old Scots pine needles and (3) a decrease in the contribution of Gln to the FAA pool in Douglas fir. The difference in Arg concentration between control and fertigation became smaller with increasing needle age. The total N concentration in the needles tended to decrease due to fertigation (Table 7.4).

Comparison of Douglas fir and Scots pine

The concentration of soluble protein and the specific activity of GS in fertigated trees did not differ significantly from the control in any of the harvests, with the exception of the February 1990 harvest of Scots pine (Fig. 7.1). Therefore, the data were pooled in order to simplify the comparison of the two tree species (Table 7.5). Pooling of the data sets showed that the concentration of soluble protein and the specific activities of GS and GDH were significantly higher in Scots pine than in Douglas fir, respectively 48, 44 and 25%.

The sum of the concentrations of Arg, Asp, Glu, Gln, His and Pro represented 77-87% (on a molar basis) of the total FAA concentration in Douglas fir and 69-85% in Scots pine.

7.5. Discussion

In the present experiment, fertigation reduced the high foliar concentration of Arg, observed in the needles of the control trees. In literature, a decrease in the Arg concentration in needles has been related to an improvement in the nutrient balance or in the water relations. Thirty-year-old Scots pine trees, fertilized annually for 17 years with N, P and K, had a significantly lower Arg concentration than trees, fertilized with the same amount of N only; viz. 15 compared with 110 μmol g^{-1} dry weight (Näsholm & Ericsson, 1990). Green Scots pine trees with significantly higher foliar K/N and P/N ratios than yellow trees, also had a significantly lower concentration of Arg in their needles; viz. 30 compared with 140 μmol g^{-1} dry weight (Van Dijk & Roelofs, 1988). Arginine was also the major component of the total FAA pool in needles of Scots pine trees, exposed to 240 μg m^{-3} NH_3 for 14 weeks (Pérez-Soba et al., 1994a); the high foliar N concentration reduced the K/N and P/N ratios (Van der Eerden & Pérez-Soba, 1992). Arginine biosynthesis increased in leaves of woody and herbaceous plants as a response to the increased NH_4^+ content of the leaves during P deficiency (Rabe & Lovatt, 1986). High levels of Arg in needles have been related to drought stress in seedlings of Pinus ponderosa (Vance & Zaerr, 1990). The foliar accumulation of Arg in conifers has also been associated with

very high foliar N concentration, far above the optimal production level, as a result of high N deposition (Pietilä et al., 1991; Van Dijk et al., 1992). Based on this literature information, we interpret the decrease in Arg concentration observed in the fertigated trees of our experiment as:

(1) an improvement in the mineral balance. The K/N and P/N ratios in the fertigated trees of the present experiment were improved and close to optimal levels (De Visser et al., 1994);

(2) a decrease in the total N concentration in the needles. Fertigated trees had a lower foliar N concentration than the control and this difference increased steadily from 1990 to 1992 in both tree species. The lower total N concentration in needles of fertigated trees was probably caused by a higher growth (dilution effect). The tree growth in those years, measured via the increase in stem diameter and basal area, was higher in the fertigated plot (De Visser et al., 1994);

(3) an improvement in the water status. On dry summer days, the xylem water potentials during day and night were approximately 0.30 MPa higher in fertigated trees than in the control, with on average xylem potential of -2.00 MPa at noon (Ten Klooster, 1993).

The improved mineral balance and water supply in fertigated trees might contribute to the one-month delay in the maximum needle fall in autumn which was observed in our experiment (De Visser et al., 1994). On the other hand, needle fall was accelerated in trees with a foliar nutrient imbalance between P and N (Aronsson, 1985). In addition, water stress accelerated needle senescence and retranslocation of nutrients from the old needles to the new foliage (Waring, 1991). The decreased Arg concentration in needles of fertigated trees was observed in 1989, only four months after the start of fertigation in the Douglas fir stand, indicating that Arg in conifers could be used as an early indicator of changes in the physiology of the tree. Kim et al. (1987) also suggested that certain amino acids, including Arg, might be more sensitive indicators of N status than total foliar N itself, as Arg responded directly to N fertilization in their experiment.

Glutamic acid was the major amino acid of the total FAA pool in conifers grown under ambient air conditions with low atmospheric N deposition (Näsholm & Ericsson, 1990; Manderscheid & Jäger, 1993). In our experiment, Arg was the major amino acid of the FAA pool in the control trees and Glu in the fertigated, with a lower N concentration. The replacement of Arg (control plot) by Glu (fertigation plot) might be due to differences in foliar N concentration. Arginine seems to store N more adequately than Glu in conditions of excess N concentration, since Arg has the highest N/C ratio of all the amino acids (4/6), compared with Glu, 1/5. The interconversion between Glu and Arg in response to a change in N status seems plausible since Glu is the common substrate of Gln, Arg and

Pro. A larger contribution of Asp to the FAA pool in the fertigated trees might be induced by the optimal supply of water since it is negatively correlated with water stress (Vance & Zaerr, 1990; Manderscheid & Jäger, 1993).

In the present experiment, the soluble protein concentration and GS activity were not significantly influenced by fertigation, neither in Douglas fir nor in Scots pine. This result agrees with the lack of effect of nitrate or ammonium levels in the soil, on GS activity and soluble protein concentration in needles of Picea abies (Manderscheid & Jäger, 1993). The activity of GS in the shoots was also unaffected by N application to the roots of Pinus banksiana seedlings (Margolis et al., 1988). The lack of effect of fertigation on these components of the N metabolism in needles might be explained by the location of the primary assimilation of the N, supplied with the fertigation. Such nitrate and ammonium, supplied to the soil, are mainly assimilated in mycorrhizas and roots rather than in the shoots of conifers (Finlay et al., 1989a).

The activity of GDH in Douglas fir needles was about 45% higher in the fertigated trees than in the control in October 1990 and 1991 (Fig. 7.1c), which could indicate sugar limitation by the greater growth of the fertigated trees. Indeed, the concentration of sucrose in the cell sap of Douglas fir needles was much lower, e.g. in October 1991 on average 5 mmol l^{-1} in the fertigated plot compared with 66 mmol l^{-1} in the control (Ten Klooster, 1993). In cell suspension cultures of carrot the highest values of GDH activity were found during sucrose limitation (Robinson et al., 1991). They observed that the activity of GDH increased by a factor 3.5 when sucrose concentrations decreased from 30.0 to 1.5 mmol l^{-1}. This correlation between GDH activity and sucrose concentration agrees with the catabolic role of GDH (Stulen, 1986), catalyzing the oxidation of glutamate thus supplying carbon skeletons for the optimal functioning of the tricarboxylic acid cycle during carbon limitation (Robinson et al., 1991; Lea et al., 1992).

In our field experiment, the specific activity of GS in the needles was 44% higher in Scots pine than in Douglas fir. The activity of GS is mainly responsible for the assimilation of NH_3 (Lea et al., 1992) and thus for its detoxification, and has been found to increase in Scots pine needles after exposure to gaseous NH_3 (Pérez-Soba et al., 1994a). This suggests that Scots pine has a higher capacity to detoxify NH_3 than Douglas fir, which might contribute to the higher vitality of Scots pine in the Dutch forests, with a relatively high NH_3 deposition (Erisman, 1991; Duyzer et al., 1992). We assume that the concentrations of atmospheric NH_3 are similar in both forest sites since the distance between them is approximately 15 km and both are located in a large for-

ested area with almost equal total N deposition.

Interestingly, the residual variance of the data on GS, GDH and soluble protein was on average 1.6 times higher in Scots pine than in Douglas fir. A higher statistical variance has been related to larger genetical variability. Large genetic variation is a fundamental determinant of adaptation and survival (Müller-Starck & Ziehe, 1992), closely associated with more tolerance to stress.

The high concentrations of N and the amino acids Arg, Glu, Asp and Gln in needles of the control trees of both trees species (this paper) and the high percentage of nitrophilic vegetation that covered the soil (De Visser et al., 1994) suggest a high atmospheric N deposition in the two forest stands of our project. The results of this field experiment confirm the important role of Arg and the activity of GS in conditions of high atmospheric N, as observed under artificial conditions in the laboratory in fumigations with gaseous NH_3. The foliar accumulation of Arg in the non-fertigated trees indicates water-stress or nutrient imbalance induced by the combination of a cation-poor and acidic soil together with an excess of the amount of added atmospheric N for growth. The significantly higher levels of GS activity in the needles of Scots pine compared with Douglas fir suggest a higher capacity to detoxify atmospheric NH_3 and could contribute to the higher vitality of Scots pine in the Dutch forests.