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The “Red” Decline of Norway Spruce or “Røde Rødgraner” – Is it Ammonium Overload or Top-Dying?

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Abstract (max. 2000 char.):

In 1989 a new disease “røde rødgraner” or “red” decline of Norway spruce (*Picea abies*) became a serious problem in plantations in Jutland on poor, sandy soils. Some trees became red and lost their needles over a few years. The reddening started from the shoot tips.

The only important pollutant in mainly rural Jutland is ammonia from the many farms, resulting in N depositions of 10-30 kg ha^{-1} $year^{-1}$. The ammonium hypothesis of Nihlgård (1985) stipulates that certain types of forest decline is caused by an overload of ammonia from the air. Hence the possibility that “røde rødgraner” was caused by nitrogen overload was investigated. A sensitive biochemical indicator of nitrogen status of conifer trees is the content of free amino acids of the urea cycle, arginine and ornithine. In pot experiments with four times overload of N as ammonium nitrate the free arginine content increased more than 100 times, up to almost 2 % of the fresh weight. Also trees suffering from phosphorus or potassium deficiency showed large increases in arginine.

Needle samples from “røde rødgraner” (taken from the $6^{th.}$ whorl at minimum metabolism in December) showed normal arginine and ornithine contents. There was no indication of nitrogen overload. The “red” Norway spruce may suffer from “top-dying” a common disorder of Norway spruce in Great Britain, believed to be caused by several mild winters in a row. In that case the symptoms should diminish after the very cold winter 1995-96.

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Contents

Introduction

Since 1989 Norway spruce (*Picea abies*) in Danish plantations, especially on poor, sandy soils in western Jutland has been seriously damaged with a complex of symptoms which in Denmark is called "røde rødgraner" or "red" Norway spruce (Yde-Andersen 1991, Saxe & Larsen 1992, Saxe 1993). This syndrome where the needles redden and die from the shoot tips inwards have resulted in more than 10 % dead trees in certain places and much premature logging. Hypotheses on the possible causes are numerous: air pollution, acid rain, N-overload from the air, drought, nutrient leaching, potassium deficiency or other nutrient imbalances, salt damage, and mild winter damage, and sums of accumulated stresses. The disease seems not to be caused by pathogens or pests. Most work in Denmark has been concerned with three hypotheses:

1) Air pollution with ammonium (Nihlgård 1985)
2) Mild winter damage
3) Combined stresses

Most damage has occurred far away from densely populated areas and removed from major sources of industrial and traffic pollution. The only important pollutant in the area is ammonia liberated from pig and dairy farming with deposition rates of 10-30 kg ha^{-1} nitrogen (Asman et al. 1998, Andersen and Hovmand 1995). One case of ammonium damage is known in chlorotic Scots pine (*Pinus sylvestris*) in the Netherlands where ammonia deposition rates are much higher than in Denmark (Van Dijk and Roelofs 1988, Pearson and Stewart 1993). Saxe (1993) analysed the contents of inorganic nutrients in “red” Norway spruce; he did not see any significant nutrient imbalance. It is known that the content of free amino acids is a sensitive measure of nutrition imbalances and a number of other environmental factors (Durzan and Steward 1967, 1983, Stewart and Larher 1980, Gezelius and Näsholm 1993).

The purpose of this work is to establish if amino acids could be used as biochemical indicators for overdoses of nitrogen in Norway spruce (Wild and Schmitt 1995). Such work has been done for other Picea species (Durzan and Steward 1967, 1983). The work will focus on the amino acids arginine and ornithine, members of the urea cycle (Aarnes et al. 1995) which are very important in amino acid metabolism and storage in the gymnosperms.

Materials and Methods

Two year old Norway spruce (*Picea abies*) were obtained from the Tree Improvement Station, Krogerupvej 21, 3050 Humlebæk, Denmark. The original seeds were from Silkeborg (Denmark) and Westerhof (Northern Germany). A preliminary experiment was cultivated with 4-year old clones made from cuttings.

The seedlings were grown in open air for two seasons in 10 L pots in very pure quartz sand, fit for glass fabrication. Ten plants were grown per pot. There were four replications per treatment. The pots were placed over 10 L reservoir flasks and equipped with an automated air-lift watering system with drainage back into the reservoir (Haahr 1975). The plants were watered twice a day for two hours. During winter the plants were kept around 5^{o}C in a greenhouse. The basal nutrient solution was designed for Norway spruce with the relations of N:K:P:Mg:Ca:S of 100:50:16:5:5:9 and 0.5 g nitrogen per pot added as ammonium nitrate (Ingestad 1979). Higher nitrogen (2, 4, and 8 fold) was given in two ways, either as additional ammonium nitrate (0.5, 1.5, 3.5g N), or as urea (0.5, 1.5, 3,5 g N) painted on the needles as a 10 % solution to simulate ammonium deposition from the atmosphere. All fertilisers except nitrogen were added at the start of the growing season. Nitrogen was given three times during shoot development from April till the end of June. All water used was deionized.

Samples for amino acids were taken during the winter to minimize diurnal and other variations associated with changes in growth phases (Durzan et al. 1983, Kim *et al* 1995). Current year needle samples from an observation plot for "red" Norway spruce decline at Feldborg skovdistrikt (also observed by Saxe 1993) were taken from the south and east branches of the 6th whorl. The samples were transported at 0-1^{o}C and kept at -18 ^{o}C until analysis.

Amino acids were extracted by disintegrating 200 mg (Ultraturrax) of current year needles and 0.4 g polyvinylpolypyrrollidone with an internal standard of norleucine in 10 ml of modified Redgwell's mixture (Redgwell 1980) of methanol:$CHCl_3$:water 12:5:3. The homogenate was centrifuged, and the precipitate reextracted with 10 ml of methanol:water 1:4. The combined supernatants were separated in two phases by adding chloroform and water, and the green chloroform phase discarded. The extracts were rotoevaporated to dryness and taken up in 1 ml water. The solution was passed through 10 ml sephadex C-25 cation exchange gel (H^+)form, washed with 30 ml of H_2O, and the amino acids eluted with 30 ml 0.2 M NH_3. The ammonia eluate was lyophilized, the residue taken up in 0.3 ml water, transferred to a dram glass and dried in vacuo. The amino acids were converted to phenylisothiocyanate derivatives according to the picotag method and analysed on a Merck-Hitachi high pressure liquid chromatograph on a 30 cm C-18 column using an acetate buffered acetonitrile gradient with EDTA (Cohen and Strydom 1988).

Four similar experiments were run. Each experiment lasted two seasons where the plants were kept in the greenhouse at 2-8 ^{o}C (as low as possible) during the winter between two seasons. An experiment was started every year for 4 years. The first experiment was preliminary, involving only N overload, using quite large trees from cuttings. The plan of the other three experiments can be read from Table 1. Trees were grown without nitrogen; with Ingestad balanced nutrients; with 2, 4 and 8 times N excess either as ammonium nitrate in the nutrient solution or as urea painted on the needles; with phosphorus deficiency, potassium deficiency, sulphur deficiency, calcium

deficiency, or magnesium deficiency; or with plants that received only nitrogen as ammonium nitrate or urea, but none of the other nutrients. The experiments also involved the micronutrients, but the results are not included because they were not different from the balanced nitrogen control.

Growth analyses involved fresh weight, dry weight, height and the needle dry weight in percent of the total above ground dry weight in order to obtain a quantitative measure of the needle loss after N overload. Only fresh weight and needle percentage are reported in the present paper.

Results

Table 1 gives the growth responses and arginine and ornithine contents of the four year old plants measured after two seasons of growth. There are very large differences

Table 1 Growth, arginine and ornithine contents in the first large pot experiment. The small trees harvested after 2 seasons, 4 years old. Arginine increased up to more than 2 per cent of the fresh weight under large N overload. Ornithine increased to 0.3 percent.

Treatment	Fresh weight Gram plant^{-1}	Arginine Gramkg^{-1} needles	Ornithine Gramkg^{-1} needles
No nitrogen added	2.8 ± 0.4	0.07 ± 0.02	0.008 ± 0.004
Balanced nitrogen (Ingestad)	11.6 ± 0.4	0.10 ± 0.04	0.009 ± 0.006
2 x nitrogen, ammonium nitrate	18.7 ± 1.6	3.8 ± 1.1	0.10 ± 0.03
4 x nitrogen, ammonium nitrate	16.8 ± 1.5	15.7 ± 2.6	2.2 ± 0.5
8 x nitrogen, ammonium nitrate	19.1 ± 2.5	23.7 ± 3.8	3.1 ± 0.6
2 x nitrogen, urea on needles	17.3 ± 1.2	1.7 ± 0.7	0.016 ± 0.007
4 x nitrogen, urea on needles	15.0 ± 1.8	14.5 ± 4.0	1.1 ± 0.5
8 x nitrogen, urea on needles	12.6 ± 2.5	20.4 ± 2.1	2.9 ± 0.7
Minus phosphorus	3.7 ± 0.3	19.0 ± 1.5	0.39 ± 0.04
Minus potassium	6.4 ± 0.7	4.2 ± 1.3	0.15 ± 0.05
Minus sulfur	10.8 ± 0.8	0.18 ± 0.04	0.013 ± 0.003
Minus calcium	9.7 ± 0.2	0.13 ± 0.07	0.015 ± 0.008
Minus magnesium	10.6 ± 0.8	0.21 ± 0.07	0.006 ± 0.002
+ N, minus all other nutrients	4.4 ± 1.2	17.9 ± 4.7	0.56 ± 0.25
+ N as urea, minus all others	2.7 ± 0.3	16.3 ± 1.6	0.56 ± 0.35

between treatments, but also very large variation in the genetically inhomogenous material. Even trees in pots minus nitrogen or + nitrogen minus all other nutrients continue growing during both seasons. The differences in arginine and ornithine contents are very large, more than 200 fold. At the highest levels more than 2 % of the needle fresh weight consists of arginine.

Table 2 gives examples of total free amino acid analyses on plants grown on the control (Ingestad balanced nitrogen), on 8 times N excess, and with phosphorus deficiency. Besides the very large increases in arginine, ornithine and lysine under both

Table 2. Amino acids in Norway spruce needles grown for two years on Ingestad nutrient solution (control), with 8 times N-overload as ammonium nitrate or with phosphorus deficiency. Large increases in arginine, ornithine and lysine.

Amino acid	8 x N overload μmoles g^{-1} fw	Control μmoles g^{-1} fw	- phosphorus μmoles g^{-1} fw
Arginine	136.0	0.60	109.0
Ornithine	23.0	0.07	2.9
Lysine	5.0	0.04	3.3
Asparagine	6.7	0.03	0.38
Proline	3.2	0.15	0.80
Glutamine	2.6	0.08	0.55
Aspartic acid	0.73	0.19	0.64
Glutamic acid	0.70	0.66	1.09
Serine	0.32	0.26	0.25
Glycine	0.02	0.11	-
Citrulline	0.02	0.01	0.01
Threonine	0.79	0.05	0.19
Alanine	0.30	0.17	0.23
Tyrosine	0.15	0.05	0.06
Valine	0.17	0.07	0.08
Methionine	0.18	0.04	0.08
Cysteine	-	0.06	0.01
Isoleucine	0.12	0.04	0.07
Leucine	0.13	0.04	0.08
Phenylalanine	0.20	0.13	0.13

N excess and P deficiency there are large increases in asparagine, glutamine and proline under N overload.

Table 3 gives the results on arginine contents in the four different experiments. The results of the N overloading results in 130-160 μmoles g^{-1} in all four experiments,

Table 3. Arginine contents compared in the four different experiments after 2 seasons of growth.

Treatment	Control μmoles g^{-1} fw	8 x N overload μmoles g^{-1} fw	- phosphorus μmoles g^{-1} fw
Preliminary experiment	0.41	162	-
First experiment	0.60	136	109
Second experiment	1.4	163	19
Third experiment	1.5	146	66

corresponding to 2.2-2.8 % of arginine in the needles. The phosphorus deficiency experiments have given more variable results.

The contents of arginine tend to build up over two seasons as shown in table 4. There is no response to potassium deficiency after the first season, but some response after the second season. The response to N excess and P deficiency is strong already after the first season, but increases even more after the second season.

Table 4. Arginine contents measured after the first and second season growth in pots.

Treatment	First season μmoles g^{-1}	Second season μmoles g^{-1}
Control (Ingestad)	0.7	1.5
8 times overload urea	52	115
Minus phosphorus	19	67
Minus potassium	1.2	4.1

Table 5 shows the ratio of needles dryweight to dryweight of the above ground biomass, expressed in percent. This is only a rough measure of needle loss, but it can be seen that N overloaded trees have fewer needles than the controls. This is because these trees had lost almost all second and third year needles.

Table 5. Reduction in the amount of needles in N-overloaded trees, expressed as needles in percent of total dry weight.

Treatment	Needles, percent of dry weight
Control (Ingestad nutrients)	46 ± 3
8 times overload N, ammonium nitrate	33 ± 3
8 times overload N, urea	31 ± 3
Minus phosphorus	37 ± 3

Figure 1 shows the arginine contents in 50 trees suffering from "red" decline of Norway spruce, compared with small trees overloaded with nitrogen. The arginine and ornithine content of the damaged trees falls within the normal range. There is no indication of the arginine and ornithine increases seen after N-overload or nutrient imbalance.

Discussion

Basically, the present work on the relation between nutrition and amino acid content confirms and extends previous results. But it does differ from the results of the many previous studies in two important ways: First, the effects of nitrogen excess or lack of potassium and phosphorus are much more dramatic in the present long-term, “chronic” pot experiments than in most other reports, except those of Durzan and Stewart (1967) and Gezelius and Näsholm (1993). The amounts of free arginine + ornithine reached more than 2 percent of the needle fresh weight under phosphorus deficiency or severe nitrogen excess. The levels obtained correspond to those found by van Dijk and Roelofs (1988) in ammonium poisoned pines in the Netherlands. Second, the study is a link between the short term work in water cultures or pots in growth chambers or greenhouses (Durzan and Steward 1967, 1983, Gezelius and Näsholm 1993, Kim et al. 1987, van Dijk *et al* 1990), ammonia fumigation (Pérez-Soba et al.1994), pollution investigations involving ammonia (Van Dijk and Roelofs 1988, Huhn and Schulz 1996), and the studies on amino acids circulation in xylem (Rennenberg et al. 1998, Schneider et al. 1996) and long term forest fertiliser experiments (Aronsson 1985, Edfast et al. 1990).

The amounts of free amino acids at severe nitrogen excess or phosphorus deficiency are so large that the plant cells must be in an abnormal osmotic state. (Table 1, Figure 1). Even at a modest nitrogen excess, two times N there was 0.2-0.4 % free arginine present after two growth seasons.

The results on arginine, ornithine and other amino acid contents were quite variable; the variance on sampling exceeded the variance on the chemical analyses. If one includes the large seasonal variations observed by e. g. Durzan and Steward (1983) and Kim and Glerum (1995) it is obvious that arginine and other amino acid contents may well be sensitive biochemical indicators of nutrient imbalances, but they are not

very precise. High levels of arginine and ornithine indicate unbalanced nutrition, but not exactly what is wrong.

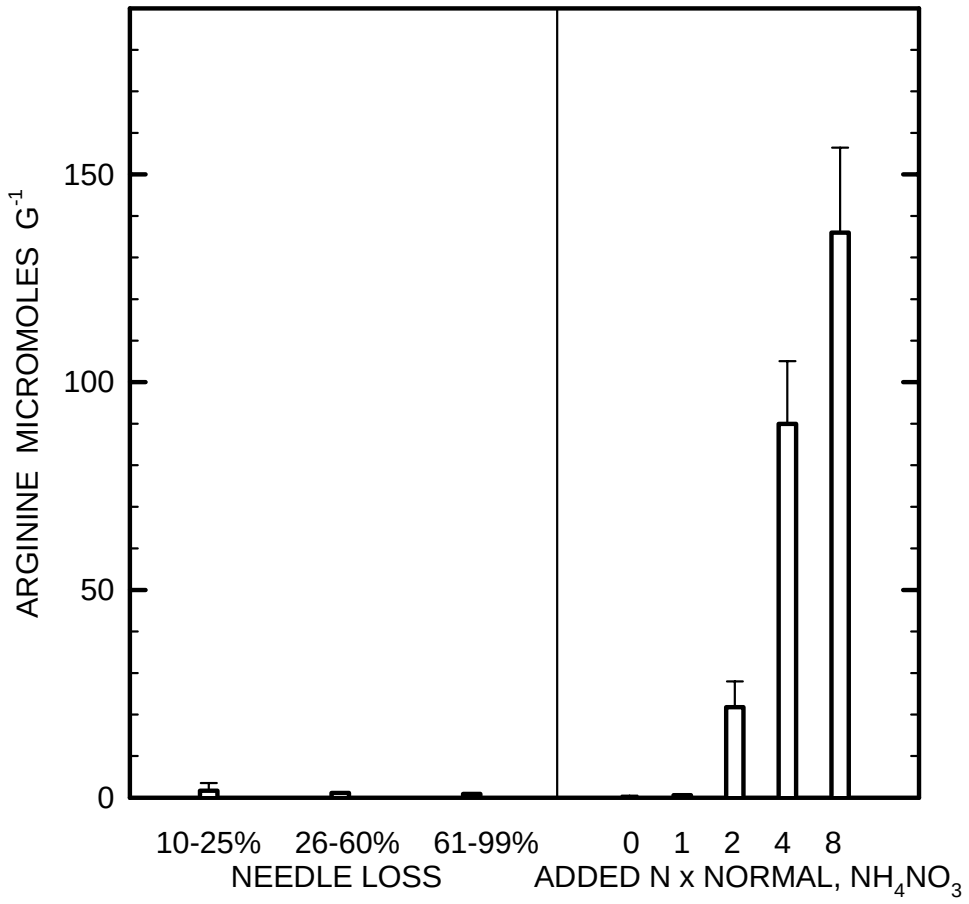

Figure 1. Arginine content in needles of Norway spruce suffering from the "red" decline or "røde rødgraner" syndrome and needle loss, compared with arginine content in needles from small trees overloaded with N as ammonium nitrate in pot cultures.

We did not see arginine and ornithine accumulation induced by deficiency of sulphur, calcium, magnesium (table 1) or the micronutrients (data not shown). It is possible that these nutrients have no influence on the free amino acid contents; but it is also possible that the 2 year old plants that were used for the experiments had already accumulated sufficiently large reserves of these compounds to last for several growing seasons.

An important part of the experiments was the amino acid contents of the dying Norway spruce in the Danish plantations in Jutland compared to the N-overloaded pot experiments. In the observation plot of the spruce in "red" decline in December 1995 there were 13 trees with mild damage (10-25% needle loss), 30 trees with medium damage (26-60% needle loss) and 7 trees with heavy damage (61-99% needle loss). About 10 trees were dead (markings has disappeared on many dead trees).

A major part of the results are given in figure 1. When one compares the low arginine contents of Norway spruce trees in "red" decline with the ammonium nitrate controls, there is no indication at all that the sick and dying trees suffer from major nutrient imbalance. This agrees with the work of Saxe (1993) who analysed the same trees for inorganic nutrients and saw no indication of nutrient imbalance. This contradicts our initial assumption that the mortality could be explained by the "ammonium hypothesis" of Nihlgård (1985). The results of van Dijk and Roelofs (1988) have shown that one will reach arginine levels above 100 μmoles g^{-1} in the needles of Scots pine in areas with heavy ammonium loads in the Netherlands and that these levels are found in diseased, chlorotic trees. Healthy Scots pine had levels below 30 μmoles g^{-1} arginine.

If the "red" decline in Norway spruce is not caused by ammonium, which is believed to be the major pollutant in rural Jutland, what might then be the cause? Barklund (1983) believe that many different factors contribute to damage and mortality: drought, acid rain causing aluminium toxicity, frost, and facultative parasites.

Another very simple hypothesis (Engvild 1996, 1998) is that the disease is identical to "top-dying" (Saxe and Larsen 1992). In Great Britain this physiological disorder actually limits the cultivation of Norway spruce (Diamandis 1978a, b, c, 1979 a, b, Murray 1953). This disease is characterised by a "spectacular browning or reddening ..... the needles redden from the shoot tips ..... the trees die from the top down over 2-3 years" (cited from Phillips and Burdekin, 1982). These symptoms are close to the ones described by Saxe (1993) and Saxe and Larsen (1992). "Top-dying" is believed to be caused by several mild, windy winters in a row, perhaps worsened by spring drought (Diamandis 1979b, Phillips and Burdekin 1982). In Denmark we have had 8 mild, windy winters in a row since 1987. The first "red" decline appeared in 1989.

If the "red" decline is indeed identical to "top-dying" of Norway spruce, then the symptoms should disappear after a couple of cold winters. "Red" Norway spruce decline may thus be moved from the field of pollution to the field of plant pathology (Kandler and Innes 1995) In the last years the symptoms of “red” spruce have been much less prevalent along with several winters with snow.

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