

Morphological asymmetry and chlorophyll fluorescence in Scots pine (*Pinus sylvestris*): responses to variation in soil moisture, nutrients and defoliation

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The effects of different levels of watering, nutrients and defoliation on two-year-old Scots pine (*Pinus sylvestris*) seedlings were examined. Two indicators of stress, chlorophyll fluorescence and morphological asymmetry, were also compared. Reduced watering and low levels of nutrients increased relative biomass and the length of the current year shoot, respectively, while both decreased current year needle biomass. In the defoliation gradient, an extensive defoliation resulted in reduced root biomass. Chlorophyll fluorescence was highest with the highest level of nutrients. In the moisture and defoliation experiments, different parts of the seedlings (current year leader vs. lateral shoot) showed significantly different fluorescence; there was no significant trend along the gradients. Both fluctuating (FA) and directional (DA) asymmetry in needle length increased significantly with increasing nutrient levels. In the defoliation experiment, both FA and DA increased with an increased percentage of defoliation. In the moisture gradient, no significant trend was found. Although the defoliation experiment confirmed that FA and DA correlate with stress intensity, the results on nutrient levels were contradictory: FA and DA increased with nutrient levels while chlorophyll fluorescence indicated no stress.

Key words: chlorophyll fluorescence, ecophysiology, fluctuating asymmetry, *Pinus*, stress

Introduction

Chlorophyll fluorescence has often been used to measure physiological stress in plants (Lichtenthaler & Rinderle 1988). The first symptoms of stress are likely to be detected in the photosynthetic electron transport system situated in the thylakoid membrane of chloroplasts. The ratio of variable fluorescence (F_v) to the maximum fluorescence (F_m) indicates the photosynthetic rate, and is about 0.832 in plants without stress (Björkman & Demming 1987). Plants subjected to various stressors have reduced photosynthetic rates. Among coniferous trees, for example, a lowered rate of photosynthesis has been found to occur with water shortages (Eastman & Camm 1995), low levels of fertilization (Strand 1997), or high air pollution (Saarinen 1993, Saarinen & Liski 1993).

Fluctuating asymmetry (FA) in bilateral characters results from developmental instability caused by stress during development. The resulting amount of asymmetry has been used to measure the severity of stress (Zakharov & Graham 1992, Møller & Swaddle 1995). The two other forms of asymmetry, viz. directional (DA) and antisymmetry, may also indicate stress (e.g. Graham *et al.* 1993a, Møller 1994). Among plants, FA has been used to indicate the strength of various environmental stressors, such as flooding (Martel *et al.* 1999), herbivory (Zvereva *et al.* 1997, Møller & de Lope 1998), chemical stress (Graham *et al.* 1993b, Tracy *et al.* 1995), or stress caused by high energy power lines (Freeman *et al.* 1993).

In this paper, we examined how different levels of watering, nutrients and defoliation affect two-year-old Scots pine (*Pinus sylvestris*) seedlings. We recorded how the variation in the three factors affect seedling growth and if the effect was similar in all parts of the seedling. We expected reduced growth with increasing stress. We also tested if the two indicators of stress, i.e. chlorophyll fluorescence and needle asymmetry, reflect stress intensity in a similar way.

Material and methods

Seedling material

Two-year-old Scots pine seedlings were grown in a greenhouse at Reposaari in Western Finland in the summer of 1998 from the middle of May to the middle of September. The stock was from a nearby nursery, mycorrhizal and of local seed orchard origin. The seedlings were planted in 2-litre pots with about 1 litre of soil. Before planting, the seedlings were gently rinsed to remove earlier soil. A basic mixture of sand and peat (Kekkilä Finnpeat M6) was used to attain 30 mg N l⁻¹ for seedlings in watering and defoliation experiments. In the nutrient experiment, the nutrient levels varied (*see below*). Nitrogen was used as a reference nutrient when calculating nutrient levels; the other nutrients varied in the same proportions. For example, in watering and defoliation experiments the soil contained N, P and K in proportions of 100:50:100, respectively.

Experiments

The effects of defoliation, soil moisture and nutrient levels were studied in separate experiments with five treatment levels. The seedlings were randomly assigned to different experiments and treatment levels. The sample size in the experiments varied from 25 to 27 seedlings (five to six seedlings per treatment level). In the green house, individual pots were placed on a table about 10 cm apart, and the pots were rotated twice a week to remove any differences in growing conditions. During the experiments, the temperature in the naturally illuminated greenhouse varied between 15 and 22 °C.

Watering experiment

Five levels of watering were applied (10%, 20%, 40%, 60% and 80% soil moisture). The

necessary amounts of water for each watering level was calculated, and made into a daily watering regime. Soil moisture was monitored with the help of control pots which were weighed daily to estimate soil moisture. In two occasions during the experiment, extra water was given to seedlings in the two lowest moisture treatments due to the loss of water potential.

Nutrient experiment

Five levels of nutrients were used (6, 13, 38, 100, and 150 mg N per pot). Two thirds of the nutrients in each treatment were given at the beginning of the experiment by mixing the required amount of peat with sand. The rest of the nutrients were mixed with water and given as part of the normal watering regime. The fertiliser mixed with water was Kekkila Superex9. At all nutrient levels, the relative proportions of N, P and K were about 100:40:100, respectively. Seedlings were grown in 40% soil moisture. Optimal soil moisture for Scots pine seedlings is considered to be 39%–46% (Puustjärvi 1973, Rikala & Huurinainen 1990).

Defoliation experiment

Five levels of defoliation were applied. The required percentage of needle fascicles (0, 40, 60, 80 and 100) was removed evenly from the whole seedling by cutting with scissors. Defoliation was performed one week after the seedlings had been planted. The seedlings were grown in 40% soil moisture.

Measurement of chlorophyll fluorescence

At the end of August, 15 current year needle fascicles were removed from each seedling. Five fascicles were randomly removed from both the upper and lower half of the current year stem,

and five from the longest shoot at the current year whorl. From some seedlings it was not possible to find five fascicles in all three parts, thus, some samples are smaller. The needles were placed in plastic bags and transported to the laboratory in dark cool boxes with constant temperature (20 °C). The chlorophyll fluorescence induction of each dark-adapted fascicle was measured within 24 hours. A sample of needles shows that the time being detached did not affect chlorophyll fluorescence (anova, the three experiments as a grouping variable, $F = 0.674$, $df = 2,22$, ns, and time as a continuous variable, $F = 0.053$, $df = 1,22$, ns).

As an indicator of photosynthetic activity, we used the ratio of variable fluorescence ($F_v = F_m - F_0$) to maximum fluorescence, F_m (Björkman & Demming 1987). Fluorescence was measured at room temperature with a pulse amplitude modulation fluorometer (PAM with accessory units PAM 102 and PAM 103, Heinz Walz GmbH, Effeltrich, Germany). Minimal (dark) fluorescence yield (F_0) was obtained on excitation with a weak measuring beam from a pulsed light-emitting diode. Maximal fluorescence yield (F_m) was determined after exposure to a 2-s saturating pulse of white light from a modified Schott fiber illuminator KL 1500. Each fluorescence curve was recorded with resolution of 12 bits, and 1600 sample points from each curve were used for the analysis. The fluorometer was controlled and the data were digitised with the help of FIP software (QA-Data Oy, Turku, Finland).

Measurements of asymmetry and seedling growth

In their natural position, needle fascicles are curved upwards and one of the needles is positioned partly above the other one. We defined the asymmetry of a needle fascicle as the difference in length or weight between the upper and lower needle.

The same 15 needle fascicles used to meas-

ure fluorescence were also used in asymmetry analyses. Before removing the fascicle from the shoot, the upper needle was marked with a pen. The difference in length between the two needles in a fascicle was measured under a microscope, and repeated after ten fascicles had been measured. The total length of the longer needle was measured with a ruler to the nearest 1 mm.

Then the needles were dried (24 h in 80 °C) and weighed individually. The weight measurements were repeated after all needles had been weighed once. The measure of repeatability (Lesels & Boag 1987) of both length and weight measurements, calculated for each tree separately, varied from 0.83 to 0.99. All measurements were made by H.R.

If asymmetry is related to trait size it has been recommended that the former should be divided by the latter (Palmer & Strobeck 1992). Since needle length asymmetry was significantly related to needle length in only a few treatment levels (Table 1), we did not divide it by trait size. However, the weight asymmetry has been divided with the mean weight of the nee-

dles in a fascicle because needle weight asymmetry correlated positively with needle weight in most treatment levels (Table 1).

In addition to the above variables, the length of the leader shoot and the biomass of all current year shoots were measured. At the end of the experiment, the biomass of roots, and the biomass of previous year and current year needles were measured. Biomass was measured after drying in 80 °C for 24 h.

Statistical analyses

To determine asymmetry as a variance (Palmer & Strobeck 1994), we have used a mixed two-way analysis of variance having needles as a random and sides as a fixed effect. With this method, it is possible to separate non-directional asymmetry from DA and test if variance due to asymmetry is significantly different from error. Of the four possible asymmetries (non-directional asymmetry or DA in needle length or weight) DA in needle weight was not significant

Table 1. Length and weight asymmetry regressed against needle length and weight, respectively, in the three experiments. '(ns)' refers to not significant result after sequential Bonferroni adjustment within treatment levels.

Level	Needle length			Needle weight		
	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>
Watering experiment						
10	0.372	1,77	ns	13.823	1,75	0.001
20	2.671	1,56	ns	1.675	1,56	ns
40	7.461	1,72	0.008 (ns)	1.345	1,72	ns
60	0.562	1,73	ns	45.841	1,73	0.001
80	0.089	1,88	ns	22.496	1,86	0.001
Nutrient experiment						
6	0.867	1,43	ns	0.657	1,43	ns
13	24.926	1,57	0.001	5.779	1,57	0.019 (ns)
38	1.638	1,72	ns	46.727	1,67	0.001
100	5.142	1,66	0.027 (ns)	11.016	1,66	0.002
150	0.642	1,66	ns	10.791	1,66	0.002
Defoliation experiment						
0	0.333	1,53	ns	8.974	1,53	0.004
30	0.005	1,53	ns	4.540	1,53	0.038 (ns)
60	9.483	1,54	0.003	23.967	1,54	0.001
80	11.734	1,72	0.001	30.194	1,72	0.001
100	3.375	1,40	ns	41.134	1,39	0.001

in most seedlings, and will not be considered further.

Since non-directional asymmetry can result from FA or antisymmetry (Palmer & Strobeck 1994), we also calculated kurtosis for each seedling. Only four seedlings showed significant kurtosis in needle length, being leptokurtic. Because leptokurtosis is unlikely to result from antisymmetry, we included all seedlings in our analyses.

The differences in needle growth and chlorophyll fluorescence within seedlings were examined with a repeated measures analysis of variance. The five treatment levels of each experiment were used as a between subjects effect, while the fascicles from the different parts of the seedling were used as a within subject effect. Only multivariate *F*-values for the within subject effect are given since the tests for sphericity were significant in some comparisons.

When analysing stress indicators against treatment levels, we have used linear regression unless the data suggest nonlinear relationship.

Results

Seedling growth

The effect of treatment levels on seedling growth was examined through multiple regression by asking if the treatment level could be predicted from the biomass of roots, needles or shoots, or from the length of the current year leader shoot.

In the watering experiment, current year needle biomass increased significantly with the increased percentage of watering (Table 2). Although shoot biomass seemed to increase with increasing moisture levels, a multiple regression indicated that, if needle biomass was controlled, there was a negative relationship between shoot biomass and soil moisture. In the nutrient experiment, current year needle biomass increased significantly with increasing levels of nutrients while the length of the leader shoot decreased (Table 2). In the defoliation experiment, root biomass decreased significantly with an increased percentage of defoliation (Table 2).

Growth of current year needles

In the watering experiment, both needle length and weight differed significantly between treatment levels (Table 3). Needle weight also differed between the three parts of the seedling (Table 3), needles in the upper part of the leader shoot being heavier than the needles on the lateral shoot (Tukey's test, MD = 85.52, $P < 0.05$). There was a significant and positive linear trend both in average length and weight of needles with increasing moisture levels (needle length: $r^2 = 0.638$; needle weight: $r^2 = 0.679$, in both $n = 26$, $P < 0.001$).

In the nutrient experiment, both needle length and weight differed amongst treatment levels as well as amongst the three parts of the seedling (Table 3). In addition, there was a significant interaction amongst the treatment levels and the three parts of the seedling. A posteriori analyses within treatment levels indicated a significant

Table 2. Seedling growth in the three experiments at different treatment levels. The dry biomass (g) of roots, current year needles and current year shoots, and the length of the current year stem (mm) have been regressed against the treatment levels. Standardised partial regression coefficient, *t*-test and its significance for each variable, and the *F*-value, its significance and the coefficient of determination for the whole model are given.

	Coefficient	<i>t</i>	<i>P</i>
Watering experiment			
Root biomass	0.344	1.895	ns
Needle biomass	0.922	4.209	0.001
Shoot biomass	-0.508	-2.646	0.014
Shoot length	0.106	0.703	ns
Model: $F = 23.469$, $df = 4, 22$, $P < 0.001$, $r^2 = 0.82$			
Nutrient experiment			
Root biomass	-0.388	-1.973	ns
Needle biomass	0.819	4.649	0.001
Shoot biomass	0.159	0.955	ns
Shoot length	-0.428	-2.957	0.008
Model: $F = 9.527$, $df = 4, 19$, $P < 0.001$, $r^2 = 0.67$			
Defoliation experiment			
Root biomass	-0.771	-3.287	0.004
Needle biomass	-0.021	0.091	ns
Shoot biomass	-0.108	-0.549	ns
Shoot length	-0.152	-0.691	ns
Model: $F = 11.288$, $df = 4, 18$, $P < 0.0001$, $r^2 = 0.73$			

difference in needle weight between the leader shoot and the lateral shoot at the lowest nutrient level (MD = 82.54 and MD = 56.16, $P < 0.05$; upper leader shoot vs. lateral shoot and lower leader shoot vs. lateral shoot, respectively). The average length and weight of the needles increased with increasing nutrient levels (needle length: $r^2 = 0.707$; needle weight: $r^2 = 0.661$, in both $n = 23$, $P < 0.001$).

In the defoliation experiment, needle weight differed significantly amongst treatment levels and both length and weight differed amongst the three parts of the seedling (Table 3). However, a posteriori comparison amongst the three parts, indicated a significant difference only in needle length between the upper leader shoot and the lateral shoot (MD = 0.707, $P < 0.05$). There was neither a linear nor a polynomial significant trend between the percentage of defoliation and needle growth (second order polynomial regression, needle length: $r^2 = 0.128$ and needle weight: $r^2 = 0.188$, in both $n = 22$, ns).

Chlorophyll fluorescence

Chlorophyll fluorescence was also examined with a repeated measures analysis of variance

(Table 3). In the watering experiment, chlorophyll fluorescence was found to be significantly different in the three parts of the seedling, and there also was a significant interaction between the watering levels and the different parts of the seedling. However, a posteriori tests within treatment levels were not significant. In the nutrient experiment, chlorophyll fluorescence differed amongst the five treatment levels, and was found to increase with increasing nutrient levels (regression: $r^2 = 0.335$, $n = 23$, $P < 0.005$). The ratio F_v/F_m was closest to the expected 0.832 with the highest nutrient levels. In the defoliation experiment, the different parts of the seedling differed significantly. The highest chlorophyll fluorescence was found in the upper needles of the leader shoot, however, a posteriori comparisons were not significant (MD = 0.012 and MD = 0.013, ns, for upper leader shoot vs. lateral shoot and lower leader shoot, respectively).

Fluctuating and directional asymmetry

FA and DA in needle length correlated positively with each other in all three experiments (Table 4). In the nutrient experiment, DA in

Table 3. Repeated measures ANOVA on differences between needle length, dry weight and chlorophyll fluorescence in the three parts of the seedling and in the three experiments. 'Treatment level' refers to the five different treatment levels in each experiment, 'Part' to needles from the upper or lower part of the leader shoot and from the lateral shoot, and 'Interaction' to the relationship between treatment level and the three parts. *F* in 'Parts' and 'Interaction' refers to multivariate results.

	Treatment level			Parts			Interaction		
	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>	<i>F</i>	df	<i>P</i>
Watering experiment									
Needle length	8.38	4,17	0.001	0.89	2,34	ns	0.94	8,32	ns
Needle weight	9.13	4,17	0.001	6.03	2,34	0.011	1.28	8,34	ns
Fluorescence	1.39	4,17	ns	10.27	2,34	0.002	2.75	8,34	0.019
Nutrient experiment									
Needle length	13.19	4,16	0.001	7.94	2,32	0.004	1.07	8,32	ns
Needle weight	8.45	4,16	0.001	30.13	2,32	0.001	3.54	8,32	0.006
Fluorescence	5.23	4,16	0.007	0.74	2,32	ns	2.17	8,32	ns
Defoliation experiment									
Needle length	2.45	4,13	ns	4.29	2,26	0.039	0.81	8,26	ns
Needle weight	9.13	4,13	0.001	6.03	2,26	0.011	1.28	8,26	ns
Fluorescence	0.91	4,13	ns	5.78	2,26	0.017	0.65	8,26	ns

needle length and chlorophyll fluorescence correlated positively.

In the watering experiment, there was no significant relationship between moisture level and needle FA or DA (Table 5). In the nutrient experiment, FA in needle length and weight increased with increasing levels of nutrients, as did DA in needle length (Table 5 and Fig. 1). In the defoliation experiment there was a weak but significant relationship between the percentage of defoliation and FA or DA in needle length (Table 5), with higher levels of defoliation resulting in increased FA or DA (Fig. 2).

Discussion

Seedling growth

All our three experiments had significant effects on some aspects of seedling growth. In the watering experiment, we found that needle and shoot biomass as well as needle length and weight decreased with decreasing watering levels. This is common with drought stress (Crawford 1982, Kozłowski *et al.* 1991). However, we did not find any decrease in growth due to excessive watering at other end of the gradient, although physiological responses to flooding include reduced root and shoot growth (Crawford 1982).

In the nutrient experiment, the highest nutrient levels resulted in increased needle biomass and needle length compared with the lowest levels. This is expected since our highest nitrogen levels were higher than required for Scots pine seedlings, where as the lowest ones were likely to result in nutrient deficiency (*see e.g.* Rikala & Huurinainen 1990, Ingestad 1979).

We found that extensive defoliation significantly affected both root biomass and needle weight. In Scots pine, old needles are an important source of nutrients for elongating shoots and new needles (*e.g.* Fisher & Höll 1991, Helmisaari 1992). If needles are removed it is likely that roots become a more important source of nutrients for the developing tissues which reduces root growth. The strong connection between needle development and the condition of the roots has been shown in an experiment with

Scots pine where roots were artificially damaged (Jokela *et al.* 1996).

Stress indicators

In Scots pine and Norwegian spruce (*Picea abies*), chlorophyll fluorescence has been shown to relate to the nitrogen content of needles. For example, the values of F_v/F_m has been shown to be higher in needles from fertilized trees when compared with needles from unfertilized ones, suggesting that nitrogen deficiency may limit the photosynthetic rate (Linder & Troeng 1980, Strand & Lundmark 1995, Strand 1997). This is because most of the nitrogen in needles is associated with proteins participating in photosynthetic reactions (Evans & Seemann 1989). Our

Table 4. Spearman rank correlations between needle asymmetry measurements and chlorophyll fluorescence. Correlations set in boldface are significant after table-wise Bonferroni adjustment.

	FA in weight	DA in length	Fluorescence
Watering experiment (<i>n</i> = 25)			
FA in length	0.210 ns	0.782 0.0001	−0.162 ns
FA in weight	—	0.262 ns	0.412 ns
DA in length	—	—	0.051 ns
Nutrient experiment (<i>n</i> = 25)			
FA in length	0.362 ns	0.893 0.0001	0.529 0.013
FA in weight	—	0.444 0.029	0.340 ns
DA in length	—	—	0.614 0.003
Defoliation experiment (<i>n</i> = 19):			
FA in length	0.003 ns	0.805 0.0001	0.212 ns
FA in weight		−0.187 ns	0.141 ns
DA in length			0.176 ns

finding that the F_v/F_m ratio increased with increased level of nutrients is consistent with this. At the highest nutrition level, the ratio was closest to 0.832 measured in plants without stress (see Björkman & Demming 1987). Thus, even the highest nutrient levels of our experiment had a positive effect on growth and photosynthesis, and cannot be considered as a form of stress.

In the moisture gradient, there was an interaction between chlorophyll fluorescence values and the different parts of seedling. This indicates that water stress was unevenly distributed within the seedling, and that some parts suffered more than the others. In our third experiment, defoliation did not seem to affect chlorophyll fluorescence although it may have increased differences between the three parts of the seedling because they differed significantly in chlorophyll fluorescence.

Roy and Stanton (1999) found that responses in terms of FA were stress-specific in wild mustard. Our results support the same conclusion in Scots pine. In addition, our results also show that different stress indicators can respond differently to the same stress factor.

In the nutrient experiment, both FA and DA increased with increasing nutrient levels. A similar pattern has been found, for example, by Lappalainen *et al.* (2000) who found that FA in birch leaves increased with fertilization levels. In addition, Martel *et al.* (1999) discovered in birch

that FA increased with excess leaf growth. It has been suggested that under moderate stress plants may have enhanced growth while also expressing increased asymmetry (see Freeman *et al.* 1993). In our data, however, the photosynthetic rate does not support the idea that the most asymmetrical plants were suffering from stress. In stead, our results suggest a more general conclusion that increased FA in plants may result from any deviations from normal growth conditions (see e.g. Lappalainen *et al.* 2000).

In the defoliation gradient, FA and DA in needle length showed the expected pattern. Scots pine seedlings with extensive defoliation grew more asymmetrical needles. FA and DA in needle length correlated positively with each other both in nutrient and defoliation experiments, i.e. in Scots pine FA and DA were related both in favourable growth conditions as well as in the presence of stress.

The moisture gradient did not show any significant trend in relation to FA or DA. Due to the low number of measured fascicles per shoot we were not able to check if there were differences in FA or DA within seedlings. For example, FA in the moisture gradient could have been following the same pattern that chlorophyll fluorescence did, i.e. drought or flooding could result in larger FA only in the most susceptible parts of the seedling.

In conclusion, our results suggest that the two stress indicators, morphological asymmetry

Table 5. Linear regression between asymmetry and the treatment levels in the three experiments. FA and DA refer to fluctuating and directional asymmetry, respectively.

	Coefficient	Standard error	t-test	P
Watering experiment (df = 1,25)				
FA in needle length	0.009	0.009	1.065	ns
FA in needle weight	0.001	0.005	0.030	ns
DA in needle length	0.002	0.007	0.330	ns
Nutrient experiment (df = 1,22)				
FA in needle length	0.031	0.005	6.098	0.001
FA in needle weight	0.006	0.002	2.325	0.029
DA in needle length	0.028	0.004	6.551	0.001
Defoliation experiment (df = 1,21)				
FA in needle length	0.008	0.004	2.089	0.049
FA in needle weight	0.002	0.004	0.682	ns
DA in needle length	0.010	0.004	2.393	0.026

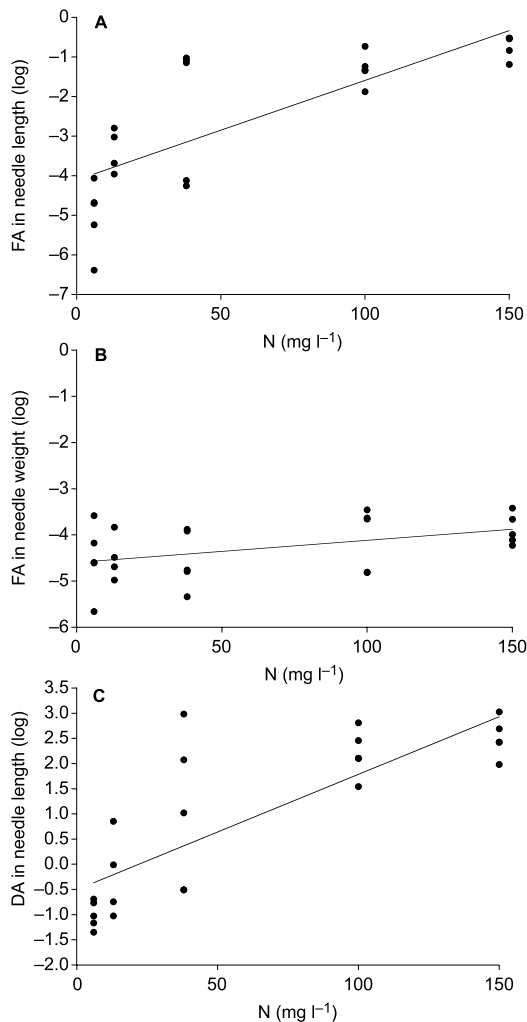


Fig. 1. Fluctuating asymmetry in needle length (A) and weight (B), and directional asymmetry in needle length (C) against treatment levels in the nutrient experiment.

and chlorophyll fluorescence, are sensitive in different ways to the variation in growth conditions. In particular, in our nutrient experiment the two indicators gave contradicting results. Our results support some earlier studies suggesting that the interpretation of FA or DA in plants may be complicated because increased asymmetry may also be combined with enhanced growth. We also found that FA and DA were closely correlated in the Scots pine, although the current data do not allow any conclusion about the basis of this correlation.

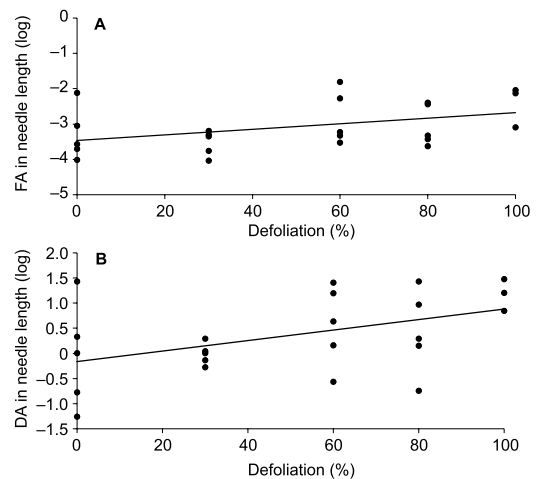


Fig. 2. Fluctuating (A) and directional (B) asymmetry in needle length against treatment levels in the defoliation experiment.

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