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Protein-thiol Groups in Spruce Needles

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With 2 Figures

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Summary

PFEIFHOFER Ch., GRILL D., PFEIFHOFER H. & ESTERBAUER H. 1988. Protein-thiol groups in spruce needles. – Phyton (Austria) 28 (1): 141–151, with 2 figures. – English with German summary.

The content of protein-bound SH groups in needles of Norway spruce (*Picea abies* [L.] KARSTEN) has been studied in relation to several local environmental factors.

Light-exposed needles have a 50–70% higher protein-SH content as compared to shaded needles. The difference between the two needle types is not markedly affected by the point of comparison i.e. fresh weight, dry weight, nitrogen content or protein content of the needles. Needles from the top of the crown also possess a higher protein-SH content than needles from the middle part of the tree. This is also likely due to differences in the light exposure. On the other hand, needles collected from 6 trees of the same age and identical environmental exposition showed very similar SH contents. In the case of a 80 year old spruce tree, the second and the third needle years possessed the highest and the fifth and sixth needle years the lowest SH content. This probably reflects the effect of aging.

The protein-SH content of spruce needles also shows seasonal variations with highest values in winter and lowest in summer. In general, over the whole year an inverse relationship exists between temperature and protein-SH content.

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Zusammenfassung

PFEIFHOFER Ch., GRILL D., PFEIFHOFER H. & ESTERBAUER H. 1988. Protein-Thiol-Gruppen in Fichtennadeln. – Phyton (Austria) 28 (1): 141–151, mit 2 Abbildungen. – Englisch mit deutscher Zusammenfassung.

In Sonnennadeln der Fichte (*Picea abies* [L.] KARSTEN) ist der Protein-SH-Gehalt um 50–70% höher als in Schattennadeln, wobei das Ausmaß der Differenz durch das verwendete Bezugssystem (Frischgewicht, Trockengewicht, Stickstoffgehalt und Proteingehalt der Fichtennadeln) nicht merklich beeinflußt wird.

Ebenfalls besitzen Nadeln aus dem oberen Kronenbereich mehr Protein-SH als Nadeln aus der Kronenmitte. Der Protein-SH-Gehalt der Nadelproben von 6 verschiedenen Bäumen war hingegen bei gleichen Standortsfaktoren und gleichem Alter der Bäume auffallend ähnlich. Bei 8 untersuchten Nadeljahrgängen einer 80jährigen Fichte war der höchste Protein-SH-Gehalt im 2. und 3. Jahrgang, der niedrigste im 5. und 6. Jahrgang festzustellen. Die Schwankungen werden mit Seneszenz in Zusammenhang gebracht.

Der Gehalt proteingebundener SH-Gruppen zeigt jahreszeitlich bedingte Unterschiede mit den höchsten Werten im Winter und den tiefsten im Sommer. Temperatur und Protein-SH-Gehalt zeigen im allgemeinen entgegengesetzte Schwankungen im Laufe eines Jahres.

Introduction

Water-soluble thiol compounds—mostly the tripeptide glutathione and, to some extent, cysteine—exhibit seasonal variation (GRILL & ESTERBAUER 1973 a, b). Glutathione is kept mainly in its reduced state (GSH) by glutathione reductase, an enzyme showing similar seasonal variation (ESTERBAUER & GRILL 1978). Cysteine is the most important metabolic thiol (RENNENBERG 1982, 1984). Glutathione is a transport and storage form of reduced sulfur, as well as a cosubstrate in various enzymatic reactions. GRILL & ESTERBAUER (1973 a) considered that water-soluble thiols play a role in a plant's response to stress. This concept agrees with the findings of LEVITT 1980. Ecophysiological studies of the influence of light on the -SH content of spruce needles (GRILL & al. 1987), studies of spruce at the tree line (HELLIG 1982, KÖLLY 1984), and of the dependence of respiration on -SH content (PFEIFHOFER & al. 1986), all point to such a role.

GRILL & al. 1982 suspect also that glutathione influences the protein-SH content of proteins. Histochemical staining indicated seasonal influences on protein-SH content (GRILL & al. 1979, 1980) but these results were semiquantitative because of interactions with phenols and unspecific adsorption to tissue particles.

The present study uses a quantitative colorimetric method with DTNB and considers a quantitative relationship to the protein content of spruce needles.

Material and Methods

We studied needles of *Picea abies* (L.) KARST. Needles from an approximately 80-year-old spruce at the edge of a south slope in Graz were used to determine the needle-year and age dependence. Specimens were taken at two-week intervals, between eight and nine o'clock in the morning, on the south side, and from the same height (4 m over ground) of the tree. The two most recent needle-years were examined immediately after the collection of samples. The influence of light and the extent of the variations in protein-SH content were performed on approximately 30-year-old spruces from the same area in Graz.

Protein-SH was measured by a modified method with DTNB described by GRILL & al. 1980. 1 g needles were homogenized in a precooled (−25°C) mortar with quartz sand (p.a.), pestle, and 5 ml precooled (−25°C) mixture of aceton-water (75:25) containing 0.15% ascorbic acid. The homogenate was not allowed to exceed 0°C. After addition of 0.5g Celite as filter aid the homogenate was sucked through a Büchner filter (−25°C) and washed with 75% acetone until the filtrate was colorless. The acetone dry powder was then suspended in 9.5 ml 6 M urea in 0.1 M phosphate buffer pH 7.0. After addition of 0.5 ml DTNB (Merck) reagent (30 mg DTNB dissolved in 10 ml 0.1 M phosphate buffer pH 7.0) the suspension was stirred for 5 minutes and then filtered. The absorbance of the clear, yellow colored filtrate was measured in 1 cm cuvettes at 412 nm (after appropriate dilution=v) using a sample without acetone dry powder as blank. From the absorbance (A) the protein SH content in micro mole per 1 g needles fresh weight was calculated according to:

$$\text{protein SH } (\mu M/g_{fw}) = \frac{A \cdot v}{1.36} \cdot$$

Preliminary studies of the same needles, with and without 6 M urea, have shown that so-called masked -SH groups (WESTHAUS & POHL 1978) make up about 40% of total protein -SH content.

As a measure of the methodic error, eight independent analyses of the same needles produced a variability coefficient of 10.9% ($\bar{x} = 3.612 \pm 0.393$).

The needles must be analyzed as soon as possible. A large number of specimens can make their conservation necessary. This can be done following the method of GRILL & al. 1988 whereby a certain quantity of needles is filled into the smallest possible glass or plastic vials and shock frozen in liquid nitrogen. After being sealed by a dense Teflon plug, the vials can be stored for up to 3 weeks at −30°C. Changes of the -SH content were within the methodic error.

Protein was measured by the Kjeldahl method (URBACH & al. 1976).

In the rule three samples were investigated. The maximum deviation extended never more than ±11%. To make the graphs better understandable we renounce to give error bars.

Temperature data were provided by the Institute of Meteorology and Geophysics at the University of Graz. Temperatures were measured for 7-day periods; the mean value over this period was entered in a temperature-time diagram on the fourth day.

Results

GRILL & al. 1988, in a study of water-soluble-SH compound content of spruce needles, have shown that needles exposed to light have a higher thiol content than those in the shade. Protein-SH shows the same tendency.

From one 30-year-old spruce, specimens were taken from either a branch completely exposed to sun or from one shaded by the tree itself or by neighbouring trees. These needles were considered light-exposed or shaded, respectively (NAPP–ZINN 1966, SCHMIDT–VOGT 1986).

Table 1

Protein-SH content of spruce needles of different exposure in relation to fresh weight (fw) and dry weight (dw).
(Standard deviation of the fw- and dw-values range from 9 to 11% of the respective value (coefficient of variability).

Needles		µmol/g fw	µmol/g dw	dw/fw	N content (%)
from the top		8.17	20.42	~0.40	1.11
mid-tree needles	light	6.10	15.64	~0.39	1.22
	shaded	2.68	7.87	~0.39	1.17
lower part of the crown	light	6.53	16.33	~0.40	1.18
	shaded	3.79	9.98	~0.38	1.18

As shown in table 1 the protein-SH content per 1 g fresh weight of the light-exposed needles exceeded that of the shaded needles by 70–80%. Similar differences are found also in relation to nitrogen or protein content of the needles, since both light-exposed and shaded needles have the same nitrogen content. This means that light exposed needles have more protein-SH groups per mg protein than do shaded needles. Similarily, the differences in protein-SH content must be interpreted as a function of the height of insertion. Needles from the top of the crown have a markedly higher protein content than mid-tree needles as it is shown in table 1. However, the approximately 25% difference in protein-SH content is not much larger than the methodic error of ±11%.

When the light conditions at the time of specimen collecting are considered, the protein-SH content of comparable trees (in age, nutrition, location, etc.) is remarkably similar. The variability coefficient ist about ±25% with an methodic error of 11%. Thus six comparable spruces had an average protein-SH of 4.43±1.09 μmol per g fresh weight.

The protein-SH content of different age spruce needles was determined from an approximately 80-year-old spruce near Graz (950 m above sea level) in August. This tree had eight needle-years. The protein-SH content of the first needle-year (1,8 μmol per g fresh weight, 4,5 μmol per g dry weight) grew by 24% and peaked in the third year (Fig. 1). It decreased after the fourth needle-year and bottomed out in the sixth needle year. The decrease between the third and sixth needle-year was 56%. The protein-SH content increased again in the two oldest needle-years.

Protein-SH content showed seasonal variation: higher in summer than in winter (Fig. 2). It was almost constant from early January to late March, averaging 4.10 μmol per g fresh weight in young needles and 4.12 μmol per g fresh weight in the older ones. In April, protein-SH in first year needles rose

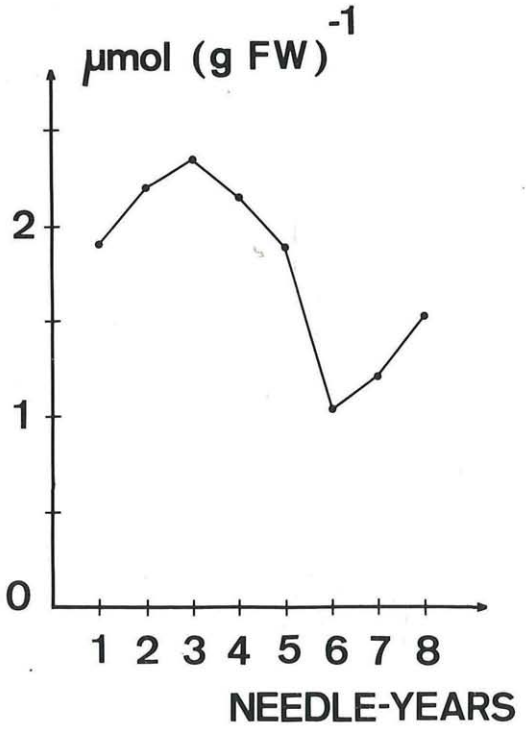

Fig. 1. Protein-SH content of different age spruce needles. Specimens were collected from an approximately 80-year old spruce near Graz in August.

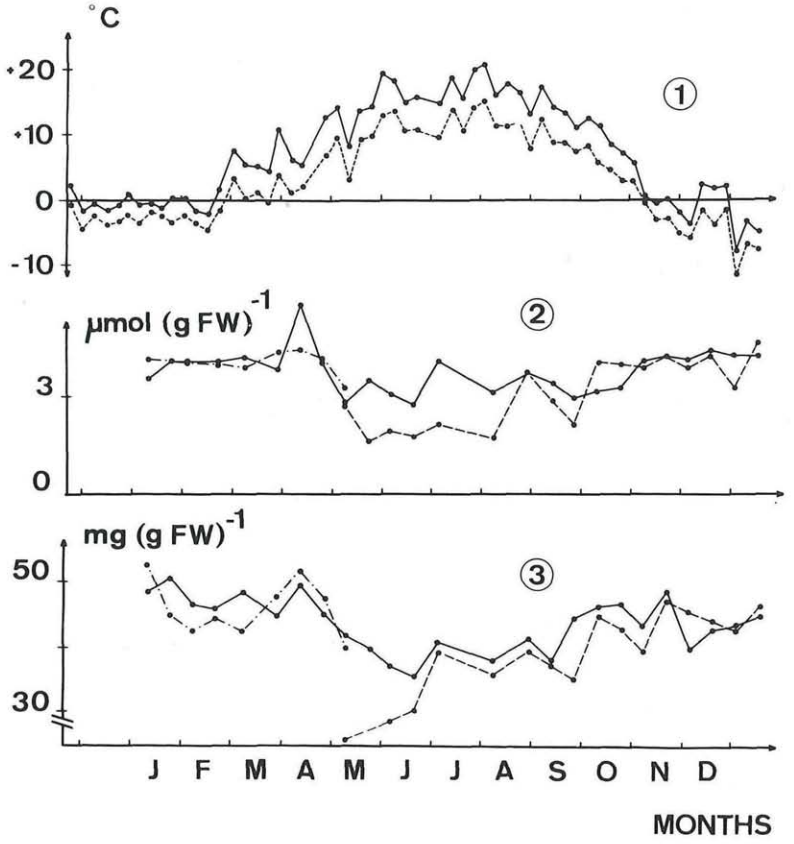

Fig. 2. Seasonal variations of temperature (1), protein-SH (2), and protein content (3) of spruce needles. Temperature was measured in the area of the Karl-Franzens-University of Graz (——— daily means, – – – – – minima). Samples for the determination of the protein-SH and the protein content were taken from a 30-year-old spruce growing on a south slope in Graz (– · – · – · = twice wintered needles, ——— = once wintered needles, – – – – – = current year needles)

by 29% (as compared to the average winter values). Then, in the wintered needles, it sank rapidly to 2.8 µmol per g fresh weight.

The protein-SH content of the newly sprouted needles (2.7 µmol per g fresh weight) was as high as that of the wintered needles. In June, as the needles developed, it decreased to 1.63 µmol per g fresh weight. In July, August and early September, protein-bound thiol content was generally lower than in winter; it averaged 3.33 µmol per g fresh weight in the younger needles but only 2.40 µmol per g fresh weight in the older ones. The content of the second needle-year fluctuated in summer; in early July it was

even comparable to winter values. The protein-SH content of younger needles was more stable. Only once, in late August, a value was recorded that lay 43% over the average summer value.

At the end of September the protein-SH content of the young needles increased and stayed high (mean 4.00 μmol per g fresh weight) until mid-January, the end of the study. An exception was seen in early January when there was a short dip of 35% compared to the average winter value. In the older needles the increase to winter levels was seen later – in mid-October. The protein-SH content of this needle-year (4.18 μmol per g fresh weight) remained practically constant until the end of the study in January.

The temperature and the protein-SH curves are inverse (Fig. 2), as can be seen also from the average values. In winter, at an average temperature of 1.8°C, the average protein-SH content was 4.00 μmol per g fresh weight in the first needle-year and 4.18 μmol per g fresh weight in the second. In summer, at an average temperature of 14.1°C, the average value was 2.12 μmol per g fresh weight in the first year and 3.17 μmol per g fresh weight in the second. In spring, the warmer daytime temperatures led to a decline of protein-SH. During the summer, the temperature minima seemed to be coupled to the protein-SH maxima: a temperature minimum was followed by a protein-SH maximum in 12–18 days. In fall, steadily cooler tempertures were accompanied by an increase in protein-SH.

The annual courses of the protein-SH content and the protein content showed similarities (Fig. 2). Both showed summer-winter variation, and newly-flushed needles showed wide variations. The protein/protein-SH ratio varied with the season and the stage of needle development. This can be followed most closely in the youngest needle-year. At the beginning of the vegetation period, the protein/protein-SH ratio was 290 (all ratios are of mass). The young needles had a low protein content but a protein-SH content almost as high as that of wintered needles. This is caracteristic of the period immediately following the sprouting of the new needle-year. It was followed by a rapid increase in protein content, while protein-SH declined.

In summer the protein/protein-SH ratio increased to 439. In Winter, after a rapid increase of protein-SH and a slower rise of protein, the ratio averaged 336. In wintered needles the ratio stayed a constant 336 – in winter and summer. It declined only in late winter, just before the new needles flushed, to 292, the same as in young needles in early May. Thus, in once wintered needles, the protein increase ist not as large as the relative increase in protein-SH groups.

Discussion

Light has been shown to have a positive influence on the water-soluble thiol content of spruce needles (GRILL & al. 1987). Protein-SH behaves

similarly. This finding holds irrespective of whether the relation is to fresh weight, dry weight, or the protein content of the needles. It is understandable in the case of the water-soluble thiols since light enhances the reduction of sulfate (RENNENBERG & al. 1979) and since the first compound formed with reduced sulfur is cysteine. Next is the tripeptide glutathione (GSH), a storage form of organic sulfur, that can be subsequently used or transported. GSH is also the dominant water-soluble thiol (GRILL & ESTERBAUER 1973 a, b, GRILL & al. 1987). However, a protein-SH increase is a result of a change in the entire protein metabolism, not of a simultaneous increase of the protein content. The protein content of the two needle types was similar, although other authors (COTHREN & GUINN 1975, LUNDERSTÄDT 1980) have observed a dependence of organic nitrogen and organic sulfur on height. GRILL & al. (1982) suspect that elevated intracellular water-soluble-SH causes the elevation of protein-SH. However, the physiologic consequences are unclear. RENNENBERG 1984 emphasized that plants try to keep their -SH content stable to avoid metabolic disturbances. This would mean that the elevanted protein-SH content of light-exposed needles has an environmentally appropriate function. However, the change of protein-SH with needle age is due to the altered protein metabolism of senescent needles. Protein-SH remains nearly the same in the first five needle-years and declines by 40% in the next two years. This agrees with KELLY & LAMBERT 1972 who reported an age-linked decline of organic sulfur in the needles of *Pinus radiata*. Increased proteolysis and nitrogen exodus can be expected in such old needle-years. The small increase of protein-SH in the eighth-needle year could be due to protein breakdown and could indicate subsequent transformation into low molecular weight, transportable forms. The old needle-years at the time of flushing are characterized by a high protein-SH content as compared to protein content.

Earlier studies (GRILL & ESTERBAUER 1973 a, b, ESTERBAUER & GRILL 1978) reported seasonal variations in the watersoluble thiol content of spruce needles. They indicated that values are lowest in summer and increase by more than three-fold in winter. Protein-SH content also shows seasonal variation. It too is higher in winter than summer, although not as much.

Other authors (BROWN & BIXBY 1973, COTHREN & GUINN 1975, MÄKINEN & STEGEMANN 1981) have indicated that temperature influences protein content and have shown that frost hardening of plants increases their protein content.

Our present study shows that protein and protein-SH have similar annual courses (–low in summer an high in winter). The difference between average summer und winter values is 15% in second-year needles and 22% in the young needles. Similar results were obtained using histochemical staining (GRILL & al. 1980). The wider variations seen in the new needle year are marked by a rapidly changing protein/protein-thiol ratio.

After flushing, protein content is low and protein-SH content relatively high, leading to a protein/protein-SH ratio of 290 (as compared to the average ratio of 439 in summer and that of 336 in winter).

The protein/protein-SH ratio changes markedly in young needles during one vegetation period, a result of accelerated protein metabolism and protein restructuring. However, the annual courses in the older needle years are almost parallel to one another.

This is illustrated also by the protein/protein-SH ratio (336) that is stable during the entire vegetation period. Thus the seasonal variations of the protein-SH content are due to fluctuations in the protein content; the increase in the youngest needles is due to accelerated incorporation of thiol groups into proteins. As with water-soluble thiols, the temporal coincidence of high -SH content and low temperature lets us conclude that it plays a role in toughening the plant for winter. According to LEVITT 1980, increased plant resistance to frost is accompanied by an increased -SH content. His sulfhydryl-disulfide hypothesis (LEVITT 1962) proposes that frost damage is a result of the oxidation of protein-bound-SH groups and the ensuing protein denaturation.

The redox system GSH/GSSG and ascorbic acid/dehydro-ascorbic acid stabilise proteins (HALLIWELL & FOYER 1978, ESTERBAUER & al. 1980) and protect the plant against frost damage. The higher winter protein-SH content—which was also demonstrated by histochemical staining—is thus closely related to the elevated GSH content of spruce needles. However, in the cellular material the protein-SH content is about ten times higher than the water-soluble thiol content. But water-soluble thiols can, in combination with substances such as ascorbic acid, protect proteins from natural stresses. Further studies showed that protein-SH values vary in winter and summer according to temperature (HELLIG 1982, KÖLLY 1984).

The concept of glutathione as a depot for the supply of young needles (RENNENBERG 1984) does not explain why light-exposed needles have more -SH than shaded needles and why trees at the treeline have more than those in the valley (HELLIG 1982, KÖLLY 1984). These facts point to stress.

In April, just before the new needles flush the protein/protein-SH ratio decreases to 292 since the protein-SH content increases more than the protein content does. That may be an expression of metabolism getting ready to supply the young needles. At the same time, the water-soluble thiols increase, glutathione (otherwise almost 100% of water-soluble thiols) declines, and substantial amounts of cysteine (over 50% of water-soluble thiols) appear (GRILL & ESTERBAUER 1973a, ESTERBAUER & GRILL 1978). After this short period, GSH resumes its normal concentration. In one-year-old needles the protein/protein-SH ratio (292) is the same as in the new needles (292).

A further fluctuation of protein-SH and nitrogen in the young needles has to be mentioned. At the end of September a small increase occurs that is

not much different than the fluctuations during the rest of the year. It is mentioned because, at this time, other metabolism systems begin to adapt to winter: ascorbic acid (ESTERBAUER & al. 1980), peroxidase (ESTERBAUER & al. 1978), chlorophyll (GRILL & al. 1983) and others. However, at the end of September the typical summer protein/protein-SH ratio and the composition of watersoluble thiols stay stable (GRILL & ESTERBAUER 1973 a, ESTERBAUER & GRILL 1978).

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