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Seasonal Variation of Glutathione and Glutathione Reductase in Needles of *Picea abies*[1]

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ABSTRACT

In spruce (*Picea abies*) needles glutathione and glutathione reductase show a periodic seasonal variation with significantly increased levels during the winter. It is proposed that glutathione and glutathione reductase play an important role for the winter hardiness of leaves from evergreen plants.

Glutathione reductase (EC 1.6.4.2) converts GSSG[2] generated by various nonenzymic and enzymic (*i.e.* dehydroascorbate reductase) reactions back to the reduced form GSH. This enzyme provides that the GSH/GSSG status of the cells is mostly in the form of GSH with only minute amounts of GSSG present. One of the main functions of GSH in plants is to protect —SH groups in enzymes and structural proteins against oxidation either by acting as scavenger for oxidizing substances or by repairing the —SH groups via the GSH-disulfide exchange reaction. The GSSG formed in both cases is reduced rapidly by the action of GR (3). According to the —SH hypothesis of Levitt (6), freezing tolerance in plants involves an increase in the resistance toward oxidation of —SH groups in proteins. Thus no formation of S—S bonds takes place when the photoplasma is progressively freeze-dehydrated. This theory, however, supposes that an effective system is operating in the leaves of frost-resistant plants (for instance spruce needles) which maintains the —SH containing proteins in the reduced state during the frost period. The results presented in this paper suggest that GSH and GR play an important role for the winter hardiness of spruce needles as both significantly increase during the cold period.

MATERIALS AND METHODS

Preparation of Enzyme Solution. The acetone dry powder of 2 g of needles (2) was shaken in 40 ml of 67 mM KH_2PO_4/Na_2HPO_4 (pH 7.4) containing 1 mM EDTA for 1 hr. The suspension was filtered through a Schleicher and Schüll No. 5893 and an aliquot part (usually 1.25 ml) of the clear filtrate was used for the enzyme assay.

ENZYME ASSAY

GR activity was measured photometrically by following the decrease of NADPH absorbance at 340 nm in a Zeiss PMQ II equipped with a recorder.

Solution A. Samples of 1 ml of 1% BSA + 1 ml of 1% EDTA + 1 ml of 0.1% NADPH were freeze-dried in small vials and stored in a freezer until use (material is stable at least for 4 months). For preparing solution A the residue of one vial was dissolved in 4 ml of 0.1 M tris-HCl (pH 7.4).

Solution B. GSSG (24.5 mg) in 10 ml of H_2O was prepared fresh each time. The test (2.5 ml) contained 1 ml of solution A, 1.25 ml of enzyme solution, and 0.25 ml of solution B. B was added last to start the reaction. Prior to addition of B the A at 340 nm was followed for 3 min to correct for any side reactions leading to a decrease of NADPH. One GR unit is defined as that amount of enzyme which catalyzes the oxidation of 1 μmol of NADPH/min. The GR activity in the needles is expressed as units/g fresh wt.

DETERMINATION OF GSH AND CYSTEINE

An aqueous extract of the needles was developed on DEAE-Sephadex A-25 and GSH and CySH were analyzed in the effluent with DTNB (5).

RESULTS AND DISCUSSION

Extraction of GR from Spruce Needles. The best way to prepare an active enzyme solution was to homogenize the needles in chilled 75% acetone and to extract the enzyme from the acetone dry powder with phosphate buffer-EDTA (2). This procedure gave reproducible results. When several needle samples from the same source were analyzed the activities/g fresh wt had a standard deviation of ±8%. Moreover, yeast GR added prior to homogenization was recovered in 85 to 110% yield, indicating that no or only minor loss of activity takes place during the preparation.

Homogenization of the needles in H_2O, 0.9% NaCl, or phosphate buffer (pH 7.4) led to a nearly complete loss of activity as shown by recovery experiments with added yeast GR. Addition of polyclar (Serva, Heidelberg), EDTA, or polyamide (Woelm, Eschwege) to the aqueous homogenizing medium did not improve the yield.

Intracellular Localization of GR. A needle homogenate prepared according to Parish (8) in a Sorbitol-Ficoll medium was fractionated by differential centrifugation. From each fraction an acetone dry powder was prepared and analyzed for GR activity; 94.5% of total activity was recovered in the 150,000g supernatant, 2.5% in 6,000g, and 0.9% in the 20,000g sediment. The 1,000g and 150,000g sediments did not contain any activity. These results suggest that the abundant part of GR in spruce needles is either not particle-bound or is released easily from organelles during homogenization. This finding is in agreement with other reports according to which most GR activity is present in the soluble part of cytoplasma (4, 7).

Seasonal Variation of CySH and GSH. We have reported

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[2] Abbreviations: GSH: glutathione; GSSG: oxidized form of GSH; CySH: cysteine; GR: glutathione reductase.

earlier (5) that the main low mol wt thiol in spruce needles is GSH which usually comprises more than 95% of the total nonprotein sulfhydryl compounds. Depending on the needle age its concentration may range from 0.07 to 0.70 μmol/g fresh wt (Tables I and II). Cysteine was found to be present only in small quantities with a concentration mostly below 0.01 μmol/g fresh wt except in April when cysteine rose for a short period up to 0.125 μmol. As shown in Tables I and II the GSH content of needles of the same age is rather independent of both the tree and the side of the tree from which the needles were collected. Remarkable differences in the GSH content were found in needles collected at different times of the year. Thus previous year needles had four to seven times higher GSH values in February than in August (Tables I and II). This finding led us to follow in detail the influence of season on the GSH content. GSH shows a characteristic seasonal rhythm with maximum concentration in winter and minimum in summer (Fig. 1). The shape of the curve in this figure would not have altered essentially if the GSH content were related to dry matter instead of fresh wt, as the dry matter of the needles shows only minor seasonal variation (Fig. 1).

Seasonal Variation of GR. Depending on the tree and the time of the year when the needles were collected the values found for GR activity ranged from 0.16 to 4.1 units/g fresh wt. Needles sampled from different sides of one tree contained rather constant levels of GR activity with a maximum standard deviation of 21%, whereas needles of the same age from different trees showed standard deviations up to 53% (Table I). In order to follow the annual variation of GR all of the samples were collected from one approximately 30-year-old spruce, from which the needles developed in 1974, 1975, and 1976 as well were analyzed for GR activity in short intervals. Like GSH the GR activity showed an annual rhythm with distinct maximum in winter and minimum in summer (Fig. 2). Although the investigations cover a period of 6 years, the position of the maximum and minimum as well as the shape of the curves for GSH and GR variation resemble each other very closely. This suggests a very effective endogenous control mechanism which guarantees that GSH and GR start to rise and fall at exactly the time every year.

Measurements of GR activity in winter and summer in the leaves of a number of other evergreen winter-hardy plants have revealed that the trend is similar in most plants studied (Table III). This suggests that the increase of GR activity in winter is a more general phenomenon not restricted to spruce.

What is the biological significance of this finding and what is the function of the high GR activity in winter? Levitt has proposed the —SH hypothesis of freezing injury and resistance (for review see ref. 6). Injury was assumed to result from protein denaturation caused by an irreversible transformation of essential —SH groups of inter- or intramolecular S—S bonds during freeze-dehydration. Freezing tolerance would then be due to prevention of intermolecular S—S bonding. It is known that GSH stabilizes protein—SH groups either by scavenging oxidizing agents or by reducing formed S—S bonds in a nonenzymic reaction. Since GSH is oxidized to GSSG in both cases, it is evident that the protecting action of GSH is effective only as long as GSSG is reduced as soon as it is formed. It seems likely that the high activity of GR and the high level of GSH in winter are protective devices which prevent the formation of S—S bonds and thus help to avoid freezing injury.

The high quantities of GSH found in the very young needles in May support the idea (1, 9, 10) that rapid growth and the juvenile state are generally associated with a high —SH content of the cells.

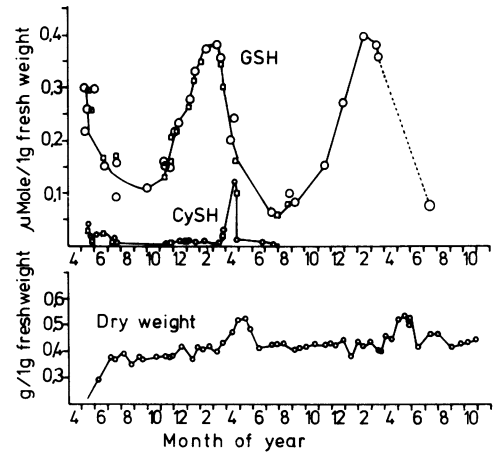

FIG. 1. Seasonal variation of CySH, GSH, and dry weight in spruce needles. CySH and GSH values were estimated in needle samples collected from different trees. For dry weight, all needles were samples from one tree. □: 1971 needles; ○: 1972 needles.

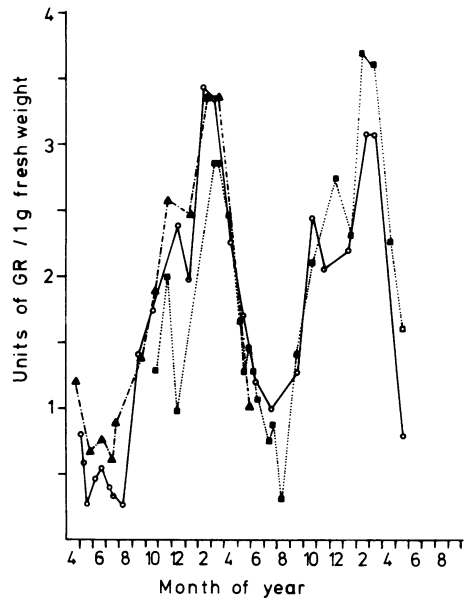

FIG. 2. Seasonal variation of glutathione reductase in spruce needles. All samples were collected from one tree. ■-----■: 1974 needles; ○——○: 1975 needles; ▲-·-·-▲: 1976 needles.

Table I. Variation of GSH and GR among spruce needles collected from eight different trees

	Age of needles Month	Date	Mean± st. dev.	Range
GSH[1]	9.5	Feb. 9	0.489±0.109	0.386 - 0.693
GSH	3	Aug.11	0.121±0.015	0.099 - 0.140
GSH	15	Aug.11	0.122±0.024	0.082 - 0.159
GR[2]	9.5	Feb. 9	3.03 ±0.96	2.00 - 4.14
GR	3	Aug.11	0.738±0.199	0.246 - 0.810
GR	15	Aug.11	0.722±0.337	0.162 - 0.907

[1] μmol per gram fresh weight
[2] units per gram fresh weight

Table II. Variation of GSH and GR in spruce needles collected from eight different sides of one individual tree

	Age of needles Month	Date	Mean± st.dev.	Range
GSH[1]	9.5	Feb.11	0.579±0.045	0.584 - 0.708
GSH	4	Aug.30	0.082±0.027	0.070 - 0.101
GSH	16	Aug.30	0.085±0.020	0.075 - 0.125
GR[2]	9.5	Feb.11	2.85 ±0.49	2.33 - 3.45
GR	4	Aug.30	0.918±0.145	0.777 - 1.03
GR	16	Aug.30	0.793±0.168	0.583 - 1.10

[1] μmol per gram fresh weight
[2] units per gram fresh weight

Table III Glutathione reductase activity during winter and summer in leaves of evergreen winter-hardy plants.

Plant	Units per gram fresh weight: current year leaves July, August	previous year leaves February	previous year leaves July, August
Picea abies (L.) Karsten	0.43	3.49	0.60
Picea omorica (Panciv) Purkyne	1.09	4.01	1.29
Picea nordmanniana (Steven) Spach.	1.16	3.36	1.23
Picea punges Engelm. var. glauca	0.38	1.87	0.45
Pinus wallichiana A.B. Jackson	0.38	1.90	1.29
Taxus baccata L.	3.60	5.34	1.88
Cephalotaxus drupacaea Sieb. u. Zucc.	1.40	4.86	1.60
Pinus mugo Turra	0.52	2.91	0.74
Chamaecyparis pisifera Sieb. u. Zucc. var. filifera	0.75	2.18	0.44
Juniperus oxycedrus L.	1.68	6.01	1.36
Juniperus chinensis L. var. Pfitzeriana Old Gold	0.97	4.75	0.84
Cryptomeria japonica (Lf.) Don.	0.90	1.68	0.90
Tsuga canadensis (L.) Carriere	0.52	1.68	0.77
Hedera helix L.	0.10	0.84	0.00
Prunus laurocerasus L.	0.06	1.55	0.02
Buxus sempervirens L.	0.70	3.49	0.32
Ilex aquifolia L.	0.25	0.13	0.01
Berberis verrucosa Hemsl. u. Wils.	0.27	3.28	0.07
Viburnum rhytidophyllum Hemsl.	0.32	0.13	0.19[1]
Ligustrum vulgare L.	0.00	0.06	n.e.
Rhododendron hirsutum L.	0.45	0.13	0.00[1]
Iberis sempervirens L.	0.87	3.11	n.e.[1]

[1] n.e. = not estimated

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