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# OCCURRENCE AND METABOLISM OF SPHAGNUM ACID IN THE CELL WALLS OF BRYOPHYTES

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**Key Word Index**—*Sphagnum magellanicum*; Sphagnaceae; sphagnum acid occurrence; metabolism; peroxidase; glyphosate; AOPP.

**Abstract**—Sphagnum acid was detected in all 30 *Sphagnum* species investigated. The content declines in older stem segments. Investigations have so far failed to detect this cinnamic acid derivative outside the Sphagnales. In all the *Sphagnum* species analysed, a second, conspicuous substance was detected, apparently identical with a degradation product of sphagnum acid produced by enzymatic reaction with peroxidase *in vitro*. A causal correlation between the sphagnum acid content and peroxidase activity *in vivo* is discussed. Glyphosate (0.5 mM) inhibits the synthesis of sphagnum acid and shikimate accumulates. Exogenously supplied phenylalanine is able to produce up to 65% reversal of the glyphosate-mediated inhibition of sphagnum acid synthesis. A mixed effect of glyphosate was found on amino acid levels. The content of sphagnum acid is also reduced by daily application of 0.1 mM L-α-aminooxy-β-phenylpropionic acid.

## INTRODUCTION

In *Sphagnum* the histochemical detection of cellulose is masked by the incorporation of phenolic compounds. On the other hand, the cell walls can be stained intense red by Millon's reagent. It is generally accepted that *Sphagnum* species do not contain lignin similar to that of higher plants [1]. The earlier concept of a primitive moss lignin is therefore no longer tenable. The incorporation of phenolic compounds occurs at rather late stages in differentiation [2]. After the extraction of these Millon's positive substances with, for example, ethanol, the detection of cellulose by histochemical methods becomes possible.

Our investigations of Czapek's sphagnol [3] clearly revealed that the isolated 'sphagnol crystals' are an artefact, namely sodium formate [4], so that today Czapek's term 'sphagnol' is only of historical interest. The positive reaction of the 'sphagnol' crystals with Millons's reagent is the result of contamination with the cinnamic acid derivative *p*-hydroxy-β-[carboxymethyl]-cinnamic acid. Masking of cellulose in the cell walls of *Sphagnum magellanicum* is due to the incorporation of this cinnamic acid into the cell walls. We isolated the cinnamic acid derivative and, referring to its origin [5], called the substance sphagnum acid. Sphagnum acid represents a natural constituent of the cell wall of *S. magellanicum*. In the present paper we report on the occurrence, distribution and metabolism of sphagnum acid in bryophytes.

## RESULTS

### *Occurrence of sphagnum acid in bryophytes*

We studied 61 bryophytes, i.e. 52 Musci, 9 Hepaticae and *Anthoceros punctatus*. In all the 30 *Sphagnum* species analysed, this cinnamic acid derivative was found in variable amounts (Table 1). The nomenclature of the *Sphagnum* species follows ref. [6]. *Anthoceros punctatus* and the following members of the Jungermanniae, Marchantiae and Bryidae were analysed: *Mylia anomala, Scapania undulata, Riella helicophylla, R. affinis, Sphaerocarpos michelii, Lunularia cruciata, Marchantia polymorpha, Riccia glauca, Leucobryum glaucum, Dicranum scoparium, D. polysetum, Cinclidotus fontinaloides, Rhacomitrium lanuginosum, Funaria hygrometrica, Tetraplodon mnioides, Tetraphis pellucida, Mniobryum wahlenbergii, Bryum pseudotriquetrum, Bryum antarcticum, Aulacomnium palustre, Fontinalis antipyretica, Fontinalis squamosa, Hookeria lucens, Cratoneuron commutatum, Pleurozium schreberi, Hypnum cupressiforme, Orthothecium rufescens, Hylocomium splendens, Polytrichum commune, Polytrichum formosum.* The nomenclature follows ref. [7]. In these bryophytes sphagnum acid was not detectable.

In every *Sphagnum* species analysed, we found, by TLC, a second substance with a bright blue fluorescence, $R_f$ 0.55. In contrast to sphagnum acid, the spot showed no significant reaction with Pauly's reagent. The substance, referred to as 'X', could be removed from cellulose by methanol and had an absorbance maximum at 312 nm.

As a rule, the collected moss material was lyophilized, ground and stored at −18°. Air drying and storing at room temperature is not a suitable method for preparing and conserving samples since, under these conditions, the content of sphagnum acid decreases continuously; because of this, sphagnum acid is often not detectable in old herbarium material.

Many bryophytes, e.g. *Sphaerocarpos, Mylia*, etc., contain phenolic compounds with $R_f$s similar to sphagnum acid and also with comparable fluorescence and quenching behaviour. These substances also show reactions with Millon's reagent and diazotized sulfanilic acid, but the UV

Table 1. Concentration of spnagnum acid in the species of *Sphagnum* analysed

| Section and species | Sites | Date | μg Sphagnum acid/100 mg dry wt |
|---|---|---|---|
| *Sphagnum* | | | |
| *S. magellanicum* Brid. | Kaltenhofer Moor | Oct. 1973 | 74 |
| *S. centrale* C. Jens. | Salla, Finn.-Lappl | Sept. 1983 | 18 |
| *S. palustre* L. | Vollstedter See | Sept. 1977 | 90 |
| *S. papillosum* Lindb. | Lebrader Moor | Sept. 1982 | 18 |
| *S. imbricatum* Russ. | Lebrader Moor | Sept. 1982 | 37 |
| *Rigida* | | | |
| *S. compactum* DC. | Trondheim | Aug. 1977 | 32 |
| *Squarrosa* | | | |
| *S. squarrosum* Crome | Vollstedter See | July 1983 | 68 |
| *S. teres* (Schimp.) Angstr. | Südtondern | Sept. 1977 | 18 |
| *Insulosa* | | | |
| *S. aongstroemii* Hartm. | Kuusamo, N. Finland | Sept. 1983 | 55 |
| *Subsecunda* | | | |
| *S. contortum* Schultz | Arlewattfeld | May 1982 | 7 |
| *S. subsecundum* Nees | Lebrader Moor | Sept. 1982 | 6 |
| *Cuspidata* | | | |
| *S. tenellum* (Brid.) Brid. | Dosenmoor | Sept. 1982 | 9 |
| *S. cuspidatum* Hoffm. | Dosenmoor | Sept. 1977 | 36 |
| *S. majus* (Russ.) C. Jens. | Kuusamo, N-Finland | Sept. 1983 | 19 |
| *S. jensenii* Lindb. | Salla, Finn.-Lappl. | Sept. 1983 | 22 |
| *S. balticum* (Russ.) C. Jens. | Kuusamo, N. Finland | Sept. 1983 | 29 |
| *S. fallax* (Klinggr.) Klinggr. | Kaltenhofer Moor | Sept. 1982 | 30 |
| *S. obtusum* Warnst. | Salla, Finn.-Lappl. | Sept. 1983 | 37 |
| *S. riparium* Angstr. | Kaltenhofer Moor | June 1983 | 22 |
| *S. lindbergii* Schimp. | Kuusamo, N. Finland | Sept. 1983 | 49 |
| *Acutifolia* | | | |
| *S. molle* Sull. | Dosenmoor | Sept. 1981 | 29 |
| *S. subnitens* Russ. & Warnst. | Lebrader Moor | Sept. 1980 | 39 |
| *S. subfulvum* Sjörs | Kuusamo, N. Finland | Sept. 1983 | 31 |
| *S. nemoreum* Scop. | Komosse | Sept. 1981 | 47 |
| *S. quinquefarium* (Braith.) Warnst. | Dolomiten | Oct. 1983 | 17 |
| *S. warnstorfii* Russ. | Vollstedter See | Sept. 1977 | 63 |
| *S. rubellum* Wils. | Wurzacher Ried | Oct. 1977 | 30 |
| *S. fuscum* (Schimp.) Klinggr. | Wurzacher Ried | Oct. 1977 | 42 |
| *S. russowii* Warnst. | Kuusamo, N. Finland | Sept. 1983 | 59 |
| *S. fimbriatum* Wils. | Vollstedter See | Sept. 1977 | 40 |

spectra are quite different. It is therefore very important to measure the absorbance maximum of the isolated substances accurately.

*Distribution of sphagnum acid in different stem segments of* Sphagnum

Seasonal variation in sphagnum acid content in the capitula of *Sphagnum magellanicum* was interpreted as a result of variations in synthesis and degradation [8]. To separate in a first approach these effects from degradation processes in stem segments, *Sphagnum riparium* was chosen as a test species. It grows rapidly under very wet conditions in pools, with the capitula just above the water surface. Overbeck [9] summarized data on measurements of increasing length; he also pointed out a higher annual growth of several *Sphagnum* species in the Kaltenhofer mire of Schleswig-Holstein than normally reported [10]. The average annual growth rate of *S. riparium* was determined as 28.5 cm; but there are also values up to 44 cm. In June 1983, individuals of *S. riparium* with an average length of 31 cm were collected from a pool in the Kaltenhofer mire. The plants were cut into five segments and the content of sphagnum acid in each segment was measured (Fig. 1). The content of sphagnum acid in the capitula was 22 μg/100 mg dry wt. The concentration in the stem segments decreased continuously, so that in the 'final brown' stem segments only 7 μg/100 mg dry wt could be determined, indicating a considerable reduction of sphagnum acid in older parts. However, these measurements on very fast growing species do not exclude seasonal influences on the values determined in different parts of the plants.

Therefore, we analysed dead and alive stem segments of *S. magellanicum* cultivated under well-defined conditions for 6 years in phytotrons. For the following segments, the sphagnum acid content was determined: the capitulum (1 cm), the following 'green' stem segment of 3 cm, 'green-brown' and 'brown' segments, 5 and 3 cm respectively (Fig. 2). The content of the capitula amounted to

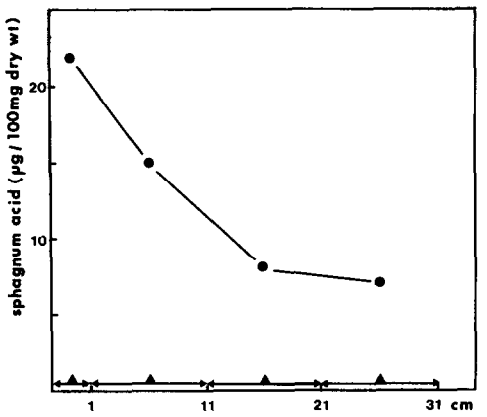


Fig. 1. Sphagnum acid content in different segments of *Sphagnum riparium*. Kaltenhofer mire, June 1983; 0–1 cm: capitula; 1–11 cm: 'green' stem segments; 11–21 cm: 'brown' stem segments; 21–31 cm: 'final brown' stem segments.

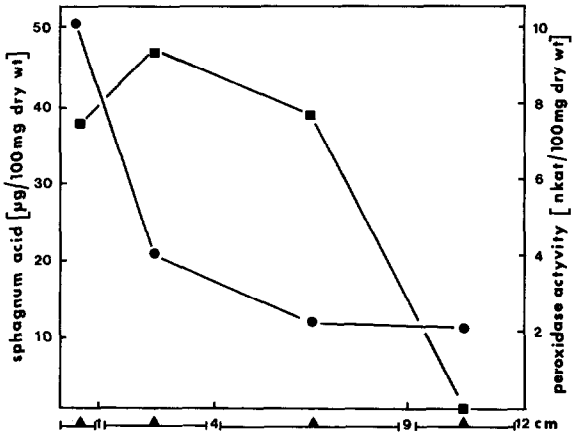


Fig. 2. Sphagnum acid content (●—●) and peroxidase activity (■—■) in different segments of *Sphagnum magellanicum*, cultivated under defined conditions in phytotrons. 0–1 cm: capitula; 1–4 cm: 'green' stem segments; 4–9 cm: 'green-brown' stem segments; 9–12 cm: 'final brown' stem segments.

51 µg/100 mg, a value in the normal range for plants grown under these conditions. In the 'green' stem segments, the value diminished to 22 µg/100 mg dry wt, a difference of *ca* 57% from the capitula.

The content declined with further decay of the plant material to 11 µg/100 mg dry wt in the 'brown' segments. Thus there is a great difference in sphagnum acid content of capitula and green living stem segments, suggesting utilization of sphagnum acid in metabolism. A further drop was observed when the cells died.

*Abbreviations: glyphosate, *N*-(phosphonomethyl)glycine; AOPP, L-α-aminooxy-β-phenylpropionic acid; PAL, phenylalanine ammonia-lyase (EC 4.3.1.5).

*Peroxidase activity in the capitula and stem segments of* S. magellanicum

We determined the peroxidase activity in different stem segments (Fig. 2). In the capitula we found an activity of 7.5 nkat/100 mg dry wt; the activity in the 'green' stem segments was higher, up to 9.5 nkat/100 mg dry wt. In the stem segments where the cells begin to die, an activity of 8.7 nkat/100 mg dry wt could still be measured. No activity was measurable in the 'final brown' segments.

*'X'—a degradation product of sphagnum acid?*

Sphagnum acid is rapidly degraded by peroxidases *in vitro* [11]. Chromatographic analysis of the incubation mixture revealed a major substance with a bright blue fluorescence in $UV_{360nm}$ light and faint fluorescence quenching of the absorbent by irradiation with $UV_{254nm}$. The $R_f$ value in *n*-butanol–acetic acid–water (3:2:95) was 0.6. We therefore incubated 44 mg of sphagnum acid with peroxidase isolated from *S. magellanicum* for 18 hr. The main degradation product was separated by preparative TLC. The UV spectrum, measured in methanol–HCl, had a maximum at 312 nm. The chromatographic data and the UV spectrum of this degradation product of sphagnum acid *in vitro* were identical with those of the substance found in the ethanol extracts of all the *Sphagnum* species analysed.

*Inhibition of sphagnum acid synthesis*

The content of sphagnum acid in *Sphagnum magellanicum* cultivated under defined conditions declined rapidly after a single application of 17 ml of glyphosate* (0.5 mM) to the capitula of 120 plants in standard vessels. In *S. magellanicum*, even with the enzymatic method proposed by Rudolph *et al.* [12], shikimate is normally not detectable, but after a glyphosate application there was obvious accumulation of shikimate in the capitula (Fig. 3).

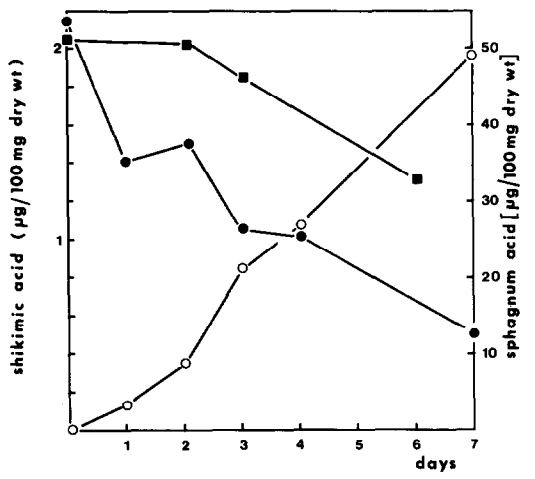


Fig. 3. Application of inhibitors. Effect of a single glyphosate application (0.5 mM; 17 ml/120 plants) on the content of sphagnum acid (●—●) and shikimate (○—○) in the capitula of *Sphagnum magellanicum*, cultivated under defined conditions. Effect of a daily AOPP application (0.1 mM; 10 ml/120 plants) on the content of sphagnum acid in the capitula (■—■).

Phenylalanine (17 ml, 1 mM) exogenously supplied to the capitula of 120 plants reversed the glyphosate inhibition by up to 65% (Fig. 4). Daily application of 10 ml of AOPP (0.1 mM) to the capitula of 120 plants also reduced the sphagnum acid content after some days (Fig. 3). These inhibitory effects, the accumulation of shikimate in *S. magellanicum* treated with glyphosate and the complementation experiment with phenylalanine indicate that sphagnum acid is synthesized via the shikimate pathway. The levels of soluble amino acids measured 24 hr after a glyphosate application were changed drastically (Table 2). In total, the content rose from 20.5 to 23.0 μmol/g dry weight. In summary, there was a mixed effect of glyphosate on the amino acid levels. The expected effect on phenylalanine could not be observed in this experiment. We prevented the flow of phenylalanine into the phenylpropanoid metabolism by the use of AOPP (0.1 mM) according to ref. [13], but glyphosate (0.5 mM) did not reduce the AOPP-mediated accumulation of phenylalanine.

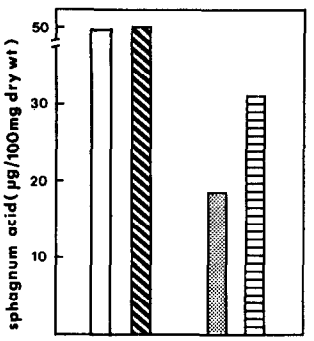


Fig. 4. Effect of phenylalanine on the glyphosate-mediated reduction of the sphagnum acid content in the capitula of *Sphagnum magellanicum*. (□) Control sample; (▤) 0.5 mM glyphosate + 1 mM phenylalanine; (▨) 1 mM phenylalanine, 17 ml/120 plants; (▩) 0.5 mM glyphosate, 17 ml/120 plants.

Table 2. Effect of 0.5 mM glyphosate on the profiles of soluble amino acids in the capitula of *Sphagnum magellanicum*

| | Amount of amino acid (nmol/g dry wt) | | |
|---|---|---|---|
| | Treatment | | |
| Amino acid | None | 24 hr after glyphosate application | 48 hr |
| Glu | 5280 | 5750 | 4080 |
| Gln | 1980 | 1050 | 1240 |
| Arg | 1840 | 1690 | 162 |
| Orn | 100 | 130 | 82 |
| Asp | 2590 | 3520 | 3520 |
| Asn | 5080 | 7100 | 4080 |
| Lys | 70 | 70 | 35 |
| Thr | 510 | 630 | 580 |
| Ile | 100 | 80 | 84 |
| Ala | 890 | 930 | 990 |
| Val | 240 | 240 | 240 |
| Leu | 170 | 210 | 110 |
| Gly | 290 | 320 | 430 |
| Ser | 1160 | 1200 | 1450 |
| Phe | 160 | 180 | 150 |
| Tyr | 70 | — | — |

## DISCUSSION

It is important to know whether sphagnum acid is generally to be found in bryophytes or is merely restricted to some classes, subclasses or even some *Sphagnum* species. Since our investigations have so far failed to find evidence for the presence of sphagnum acid outside the Sphagnales, sphagnum acid is therefore an appropriate name for *p*-hydroxy-β-[carboxymethyl]-cinnamic acid. When interpreting the data of Table 1, it is important to recognize that the samples were collected at different times during the year and seasonal variations of the sphagnum acid content have been measured. In addition to this screening programme, we studied the distribution of sphagnum acid in different parts of *Sphagnum* plants. The content diminishes in older parts of the plants where surprisingly high peroxidase activities were also found. In all the *Sphagnum* species analysed we found a substance, 'X'. We hold the view that 'X' and the degradation product of sphagnum acid are identical. Since sphagnum acid is available by synthesis [14], the peroxidase degradation of sphagnum acid would be an appropriate method of preparing 'X' in greater amounts for further analysis. Glyphosate is a specific competitive inhibitor of the shikimate pathway enzyme 3-phosphoshikimate-1-carboxyvinyltransferase (EC 2.5.1.19). This non-selective herbicide inhibits the production of phenylpropanoid compounds for which phenylalanine serves as the precursor (e.g. refs. [15–18]). The present experiments with glyphosate and AOPP substantiate the working hypothesis that the cinnamic acid derivative sphagnum acid is synthesized via the shikimate pathway.

The question remains: does the sphagnum acid represent an end product of aberrant biosynthetic pathways, producing further evidence for the systematically isolated position of *Sphagnum*, or does this cinnamic acid derivative, together with other phenolic cell-wall constituents, serve as a substitute for lignin, since $^{13}C$ NMR spectroscopy revealed $^{13}C$ signals of *p*-hydroxyphenyl residues. However, the typical lignin signals for the *β-O-4-* and phenylcoumaran structures were lacking [1]. The high content of sphagnum acid and other phenolic compounds in the cell walls of *Sphagnum* favours the view that these compounds may be partly responsible for the apparent resistance to decomposition shown by *Sphagnum* tissues.

## EXPERIMENTAL

The bryophyte samples were lyophilized, ground and stored at −18°. For separation by TLC, the conc. extracts were applied to cellulose $UV_{254}$ layers and chromatographed in *n*-BuOH–HOAc–$H_2O$ (3:2:95). Photometric determination of sphagnum acid was carried out by the method of ref. [8]. For calculation in MeOH–37% HCl (99:1), $\lambda_{max}$ nm (log ε): 269 (1.76) was used.

Enzyme preparations were carried out at 4°. Using $Me_2CO$ powder for peroxidase isolation, we followed the technique described in ref. [11]. We also extracted peroxidase from fresh material as follows: after squeezing the adhering capillary $H_2O$ with filter paper and determining the fresh wt and in sub-samples the dry wt, the plant material was homogenized with a 6-fold wt

of phosphate buffer, pH 5.0. The homogenate was filtered and solid $(NH_4)_2SO_4$ was added to the crude extract to 85% saturation. The ppt. was separated by centrifugation for 30 min at 40 000 *g*, dissolved in 5 ml phosphate buffer and desalted by the Sephadex G 25 centrifugation technique. The enzyme activity was determined by the oxidation of guaiacol in phosphate buffer (pH 5.0) after adding $H_2O_2$, a test based on ref. [19]. *Sphagnum magellanicum* was cultured under defined conditions in phytotrons according to ref. [20]. For feeding experiments, glyphosate, AOPP and phenylalanine were administered in aq. solns (17 ml on the surface of *ca* 120 plants). Free amino acids were extracted according to ref. [21]. For determination we used the amino acid analyzer LC 6000 (Biotronic); the separation program is described in ref. [22].

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## REFERENCES

1. Nimz, H. H. and Tutschek, R. (1977) *Holzforschung* **31**, 101.
2. Tutschek, R., Rudolph, H., Asmussen, L. and Altena, U. (1978) *Rev. Bryol. Lichenol.* **44**, 319.
3. Czapek, F. (1899) *Flora* **86**, 361.
4. Rudolph, H. (1972) *Biochem. Physiol. Pflanz.* **163**, 110.
5. Tutschek, R., Rudolph, H., Wagner, P. H. and Kreher, R. (1973) *Biochem. Physiol. Pflanz.* **164**, 461.
6. Isoviita, P. (1966) *Ann. Bot. Fenn.* **3**, 199.
7. Frahm, J.-P. and Frey, W. (1983) *Moosflora.* Ulmer, Stuttgart.
8. Tutschek, R. (1979) *Z. Pflanzenphysiol.* **94**, 317.
9. Overbeck, F. (1975) *Botanisch-geologische Moorkunde.* Wachholtz, Neumünster.
10. Sonesson, M., Persson, S., Basilier, K. and Stenström, T. A. (1980) *Ecol. Bull. (Stockholm)* **30**, 191.
11. Tutschek, R. (1979) *Phytochemistry* **18**, 1437.
12. Rudolph, H., Krause, H.-J., Blaicher, M. and Herms, E. (1981) *Biochem. Physiol. Pflanz.* **176**, 728.
13. Tutschek, R. (1982) *Planta* **155**, 301.
14. Wächter, D. and Rudolph, H. (1984) *Z. Naturforsch. Teil C* **39**, 311.
15. Amrhein, N., Deus, B., Gehrke, P. and Steinrücken, H. C. (1980) *Plant Physiol.* **66**, 830.
16. Amrhein, N., Deus, B., Holländer, P., Schab, H., Schulz, J. and Steinrücken, HC. (1981) *Proc. Plant Growth Regul. Soc. Am.* **8**, 99.
17. Holländer-Czytko, H. and Amrhein, N. (1983) *Plant Sci. Letters* **29**, 89.
18. Steinrücken, HC. and Amrhein, N. (1980) *Biochem. Biophys. Res. Commun.* **94**, 1207.
19. Bergmeyer, H. U. Hrsg. (1974) *Methoden der Enzymatischen Analyse.* Verlag Chemie, Weinheim.
20. Rudolph, H. (1978) *Bryophytorum Bibliotheca "Cramer" Vaduz* **13**, 279.
21. Thönes, S. and Rudolph, H. (1983) *Telma* **30**, 201.
22. Gabriel, A. (1983) Doctoral Dissert., Christian-Albrechts-Universität zu Kiel.