Ergosterol as a Biochemical Indicator of Fungal Infection in Spruce and Fir Needles from Different Sources

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Spruce Decline, Fungal Infection, Ergosterol

Different fungi are discussed to play a certain role in recent spruce decline in addition to different abiotic factors such as SO_2, photooxidants (ozone) or unfavourable climatic conditions. Thus, ergosterol as a specific fungal sterol was used as a biochemical indicator for fungal infection in differently damaged spruce and fir needles. With its aid it could be demonstrated that the important needle pathogens *Rhizosphaera kalkhoffii* Bubak, *Lophodermium piceae* and *Sirococcus* Type II contain unsaponified ergosterol comparable with the ergosterol content of different edible mushrooms. Furthermore it could be shown that all necrotic and also certain bleached and green needles exhibit fungal infection. However, fungal invasion seems to be a secondary reaction after the needle is predamaged by different factors.

Introduction

About ten years ago a mysterious fir decline was reported starting in the Bavarian and the Black Forest. The oldest needles became yellow and finally necrotic shortly before abscission. Similar symptoms were also observed for spruce in the last years. Especially during the hot summers in 1982 and 1983 the severity of the visible symptoms was strongly increased. Today more than 50% of the tree population in the forests in Baden-Württemberg and in Bavaria exhibit visible damage.

A great number of investigations were started during the last years concerning the current spruce decline. Besides SO_2, acid rain [1], photooxidants [2–6] or their combination as abiotic factors causing visible damage, different pathogens are discussed to play a certain role in spruce decline [7]. However, the discussion has been controversy [8, 9].

In most cases needles are cultured according to Rack and Butin [10] in order to get information of fungal infection. We used ergosterol, a sterol specific to fungi as a biochemical indicator for fungal infection of differently damaged spruce and fir needles. Seitz *et al.* [11] in 1977 were the first to use ergosterol as an indicator for fungal infection in grains. Several papers were published by Seitz and coworkers concerning ergosterol as a biochemical indicator for fungal infection or fungal growth in different plant material [12–14]. Similar experiments were also performed by Cantone *et al.* [15] and Lee *et al.* [16]. Our intention was to transfer this method to spruce needles in order to get more information on the contribution of fungi for spruce decline.

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Experimental Procedures

Materials

The HPLC system (Beckman, München) consisted of a one-wavelength detector (type 160), one pump (type 114), an organizer (type 340), a controler (type 420) and a printer (type C-R1A) in combination with a ULTRASPHERE-ODS 5 µcolumn (4.6 × 250 mm).

Methanol for the HPLC analysis was purchased from Promochem, D-4230 Wesel.

Touluene, petroleum benzine, diethyl ether, acetic acid and TLC-plates (silica gel 60, 0.2 mm) were acquired from Merck, Darmstadt.

Ergosterol was purchased from Sigma, München, and Malzin from Diamalt, München. For all SEM-pictures a Kontron scanning electron microscope (Eching, München, Type Jeol 35c) was used. The chlorophyll determination was performed using a Kontron UVIKON 810 spectrophotometer (Eching, München).

All needles used for the standard curve were collected from one tree in a forest near Munich (Neuherberg). This tree exhibited symptoms as described by Rehfuess and Rodenkirchen [8].

The other spruce needles were collected from different locations in the Lower Alps (Wolfratshausen, Geretsried, Bad Tölz, Marienstein and Elbach). One

sample was taken in the Spessart (Rieneck). The fir needles were from the Bavarian Forest (Mauth). All samples were taken in August and September in 1983.

Methods

Collecting spruce and fir needles

Small branches from spruce and fir were frozen in liquid nitrogen immediately after cutting in the forest. The needles were picked off and transported in dry ice and stored in a deep-freezer at −80 °C.

Cultivation of different forest pathogens

Authentic cultures of *Rhizosphaera kalkhoffii* Bubak, *Lophodermium piceae* and *Sirococcus* Type II were a gift from Prof. Butin (Institut für Pflanzenschutz im Forst, Braunschweig). The different fungi were cultivated in fluid Malzin medium (3% Malzin) for eight days in the dark at 22 °C. After washing the mycel four times with "aqua bidest." it was lyophilized and stored in a deep-freezer at −80 °C.

Extraction and determination of ergosterol from spruce and fir needles

Needles were frozen in liquid nitrogen and powdered in a coffee mill. An aliquot was dissolved in chloroform for ergosterol determination according to Höll and Goller [17], slightly modified by substituting benzene for touluene. Recently Naewbanij *et al.* [18] published a similar method using thin layer chromatography and high pressure liquid chromatography for the determination of ergosterol. Ergosterol was used as the standard for monitoring the separation of free sterols on TLC-plates. After the plates were developed in petroleum benzine (b.r. 40−60 °C)-diethyl ether-acetic acid (90:10:1, v/v) in two separate runs, the ergosterol standard was visualized by spraying with sulfuric acid−acetic acid (1:1, v/v) and heating (5 min at 120 °C). Unsprayed areas corresponding to ergosterol were scraped off and eluted with 5 ml chloroform, subjected to membrane filtration (pore size 0.2 µm), evaporated to dryness and redissolved in 80 µl ethanol. Before HPLC-separation the samples were centrifuged in an Eppendorf centrifuge for five minutes at highest speed. Ergosterol was detected at 280 nm using a 91% methanol-water mixture as the mobile phase. The identification of the ergosterol peak was done by cochromatography of authentic ergosterol. 5 µl samples were subjected to HPLC-separation.

Scanning electron microscopic pictures

Spruce needles were dryed at 28 °C, attached onto an object holder and sputtered with gold for 30 min. The gold layer was about 200 Å thick.

Needle dry weight

The needles were frozen in liquid nitrogen before all preparations and powdered in a coffee mill for two minutes. After this time an aliquot was weighted and dryed at 60 °C for three days.

Chlorophyll content

The frozen needle powder was used for chlorophyll determination according to Arnon [19].

Results

1. Scanning electron microscopic pictures of necrotic spruce needles

A great number of SEM-pictures were taken from needles of differently damaged spruce trees of the Lower Alps region. We very frequently saw typical reproductive structures in the surface of partially or completely necrotisized needles. In Fig. 1 typical pycnidia of *Rhizosphaera kalkhoffii* Bubak are

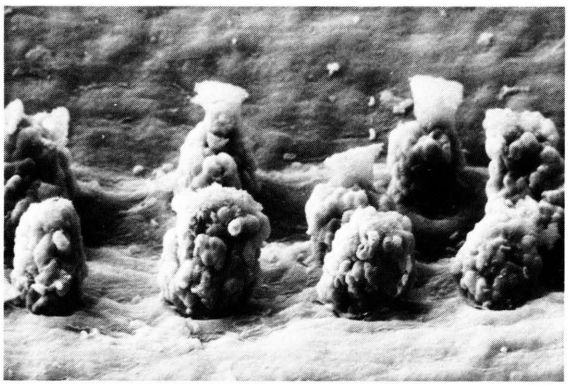

Fig. 1. Pycnidia of *Rhizosphaera kalkhoffii* Bubak breaking through the stomata of a uniformly necrotic "1983 spruce needle". The wax plugs are on top of the pycnidia (magnification ×430).

breaking through the stomata, carrying the "wax plug" on top, are visible.

2. *Ergosterol content of different forest pathogens*

All three tested forest pathogens showed significant ergosterol contents (Table I). However, *Rhizosphaera kalkhoffii* Bubak exhibited a 2.5-fold higher ergosterol level as compared to *Lophodermium piceae* or *Sirococcus* Type II.

Table I. Ergosterol content of three forest pathogens grown in 3% Malzin medium for 8 days at 22 °C.

Pathogen	Ergosterol content [μg/gdw]
Rhizosphaera kalkhoffii Bubak	255
Sirococcus Type II	119
Lophodermium piceae	95

The experiment was repeated three times with essentially identical results.

3. *"Standard curve" of the ergosterol content of samples of differently infected spruce samples*

Samples of green and necrotic spruce needles from the same tree were mixed and both ergosterol and chlorophyll contents were determined (Fig. 2). The ergosterol level of the different samples increased linearly with the content of necrotic needle portion, while the chlorophyll level decreased correspondingly. Surprisingly quite green needle samples also contained some ergosterol.

4. *Determination of the ergosterol content of differently damaged spruce needles*

All necrotic spruce needles from the Lower Alps region exhibited ergosterol levels between 1824 and 3332 ng/gdw (Table II). An approximately 10-fold higher ergosterol content could be found in necrotic fir needles collected in the Bavarian Forest. However, the highest ergosterol level was found in necrotic spruce needles from the Spessart region.

Ergosterol could also be detected in bleached and in some quite healthy looking green spruce needles. The ergosterol content of these samples was much lower as compared to necrotic needles.

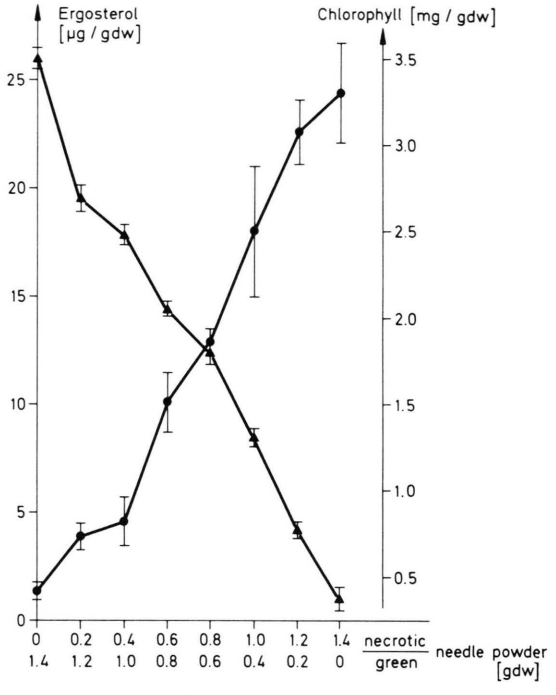

Fig. 2. Ergosterol standard curve of differently infected spruce needle samples. (The values represent means of six replications.) ●——● Ergosterol content; ▲——▲ chlorophyll content.

Discussion

In this report it is demonstrated that the three forest pathogens *Rhizosphaera kalkhoffii, Lophodermium piceae* and *Sirococcus* Type II contain unsaponified ergosterol. The ergosterol contents are in good agreement with the ergosterol levels of different edible mushrooms [20]. Furthermore the method for ergosterol detection described by Höll and Goller [17] could be transfered to spruce needles. With a slightly modified method the ergosterol content of different needle fractions could be determined. The standard curve shows an invers linear correlation to the chlorophyll content. With its help one can quantify fungal infection of spruce needles to a certain degree.

Table II demonstrates that all necrotic needles exhibit fungal infection, characterized by the highest ergosterol levels. However, great differences in the ergosterol content could be found between different necrotic needle samples. Ergosterol could also be found in some bleached needles. But they show much smaller ergosterol contents as compared to

Table II. Ergosterol content of differently damaged spruce and fir needles.

Location	Needle age group	Symptom	Chlorophyll [mg/gdw]	Ergosterol [ng/gdw]
Wolfratshausen	1983	green	3.1	0.0
	1982	faint green	2.0	0.0
	1981 and older	necrotic with black dots	0.0	3332.0
Geretsried	1983	green	3.4	0.0
	1982	yellow	0.8	0.0
	1981 and older	necrotic	0.0	2932.0
Bad Tölz	1983	yellow	1.0	0.0
	1982	green	2.8	0.0
	1981 and older	necrotic with black dots	0.0	1824.0
Marienstein	1983	yellow	0.6	108.0
	1982	faint green	2.0	0.0
	1981 and older	faint green	1.9	70.0
Elbach	1983	yellow	0.8	52.0
	1982	faint green	2.3	0.0
	1981	green	3.2	142.0
	1980 and older	necrotic	0.0	2350.0
Rieneck	1980 and older	necrotic	0.0	52678.0
Mauth (fir needles)	1982	necrotic with black dots	0.0	13650.0

The extraction was repeated twice with essentially identical results.

necrotic ones. This result can stand for the theory that in most cases fungal invasion in spruce needles will be a secondary effect. The needles can be penetrated by the pathogens after being predamaged for example by abiotic factors. This may result in a partial decrease of structural resistance as recently described by Elstner *et al.* [5]. Fungal invasion may then accelerate needle death. Similar results were obtained by Kowalski and Lang [21], who cultivated a great number of fungi from healthy looking and differently damaged spruce needles on agar.

It was surprising that in two cases green needles also exhibited a small ergosterol content. These needles may be invaded by the Ascomycet *Lophodermium piceae* a fungus which is known to penetrate only the youngest needle generation without exhibiting any visible symptoms over a long period [9].

It might be helpful to use ergosterol as a biochemical indicator for fungal invasion a) because of the high sensitivity of this method and b) in order to get an information about the population dynamics of the epidemic infection. Needles in addition should be cultivated according to Rack and Butin [10] in order to get an information about the nature of the fungi colonizing the needles.

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