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The use of ergosterol in the pathogenic fungus *Bipolaris sorokiniana* for resistance rating of barley cultivars

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Abstract

Ergosterol content in the plant pathogenic fungus *Bipolaris sorokiniana* was determined in different matrices including mycelium, spores, culture filtrate and infected barley leaves. Ergosterol was extracted with methanol, hydrolysed with KOH and quantified by reverse phase high performance liquid chromatography (HPLC). Our procedure was used to study how the ergosterol concentration of *B. sorokiniana* varied due to fungal age and nutrient availability when growing in liquid medium. It was found that the ergosterol content decreased with fungal age. The decrease was not due to leakage. It was also found that a change to a less nutrient-rich medium caused an increase in ergosterol content whereas a change to a rich medium led to a decrease. The procedure was also used for quantification of fungal infections in complex matrices (e.g. leaves). The development of fungal infection in barley leaves was followed during 10 days. Visual grading of leaf spots was also compared to ergosterol content in three varieties of barley. The ergosterol content in the leaves increased exponentially until day 7, and the grading of the leaf spots was correlated to the ergosterol content. Our results show that, despite a great variation, ergosterol may be used as a biomarker to detect and quantify fungal infections in a given matrix.

Introduction

Bipolaris sorokiniana (Sacc. in Sorok.) Shoem (syn. *Helminthosporium sativum*, Pamm. King and Bakke), a fungal pathogen of barley (*Hordeum vulgare*) causes common root rot, necrosis and chlorosis in leaves (leaf spot disease). The fungus causes severe yield losses in many countries (Piening, 1973; Stick, 1982; Kurppa, 1985). Different cultivars of barley vary in resistance towards the fungus. Currently, disease resistance is frequently assessed using visual grading of symptoms seen on the plant. Sometimes this will give a false value of resistance, for instance when infection is present but symptoms are not yet expressed, or will not be expressed (Gretenkort and Ingram, 1993). To facilitate detection of infection in plants, ergosterol, a fungal membrane specific component, can be used as a biomarker (Griffiths et al., 1985). The conjugated double bonds at positions 5 and 7 in the ergosterol molecule permit sensitive assay by measurement of the A_{282} of UV-light in high performance liquid chromatography (HPLC).

The early studies of ergosterol by Seitz et al. (1977, 1979) have been followed by a number of papers in which the authors basically use the same extraction technique (Gordon and Webster, 1984, 1986; Matcham et al., 1985; West and Grant, 1987; Nout et al., 1987; Salmanowicz and Nylund, 1988; Newell et al., 1988; Martin et al., 1990; Gessner et al., 1991; Gretenkort and Ingram, 1993; Gessner and Chauvet, 1993). These papers describe extraction of ergosterol from organic matrices including the following steps: 1) homogenisation in methanol or ethanol, 2) saponification in an alcoholic base and 3) partitioning and extraction of ergosterol from the (basic) alcohol into a low polarity solvent by the addition of water and either light

petroleum, hexane or pentane (see Gessner et al., 1991 for evaluation of different extractants). Other extraction methods involve refluxing in alcohol (Newell et al., 1988; Gessner et al., 1991; Liljeroth et al., 1993), direct extraction/saponification in an alcoholic base (Gessner et al., 1991), extraction with chloroform/methanol (Matcham et al., 1985; Schmitz et al., 1991), extraction with hexane/methanol with or without an added base (Davis and Lamar, 1992) or supercritical fluid extraction (Young and Games, 1993). Methods of pre-cleaning ergosterol samples include filtration and/or fractionation (Gordon and Webster, 1984, 1986; Salmanowicz and Nylund, 1988; Martin et al., 1990; Schmitz et al., 1991; Davis and Lamar, 1992; Snijders and Krechting, 1992).

Some of the steps used during extraction, such as homogenisation, filtration and fractionation, are time-consuming and increase the risk of contamination of the samples. We have found that for some easily extractable matrices, e.g. leaves, these steps can be excluded if refluxing is used during extraction. We have used this method to investigate how the ergosterol concentrations in the fungus vary in response to fungal age and nutrient availability and have evaluated the usefulness of ergosterol measurements for quantification of fungal infections in complex matrices. This method was applied to barley leaves infected with the fungus *B. sorokiniana* and measurements of ergosterol content were compared with visual grading.

Materials and methods

Chemicals and solvents

The solvents used for extraction and chromatography were obtained from Fisons (Loughborough, UK) and were of HPLC grade. Ergosterol and 7-dehydrocholesterol, used as an internal standard (IS), were purchased from Sigma (St Louis, MO, USA). Other solvents and chemicals were of analytical grade and not purified prior to use.

Fungus and plant material

The fungus *B. sorokiniana* was obtained from diseased barley (cv. Tellus; Svalöv-Weibull AB, Sweden) and was maintained on agar slants in screw cap test tubes at 4 °C. *B. sorokiniana* was grown on defined basal medium for spore production (Skoropad and Arny, 1957). Cultures, 15 days old and grown at room temperature (22 °C) were washed with sterile distilled water and hyphae were removed by filtering the suspension through a nylon screen (100 μm). The conidi were freeze-dried overnight. Three cultivars of barley were obtained from Svalöf-Weibull AB, Sweden, with different resistance rankings to *B. sorokiniana* infection; Berolina (most resistant), Sv 88777 (medium resistant) and Ca 602202 (susceptible).

Variation in ergosterol due to fungal age and different nutrient concentration

Three series of conical flasks (A, B and C, each series consisting of 18 flasks) were used, each flask containing 15 ml of defined liquid medium (Carlson et al., 1991) of different concentrations (100%, 1% and 0.01% respectively). The flasks were autoclaved at 105 °C for 40 min after which 125 μl of a separately autoclaved phosphate buffer (K_2HPO_4 0.75 gl^{-1} + KH_2PO_4 0.75 gl^{-1} was added to each flask in series A. In series B and C, 1% and 0.01% of the buffer solution was added, respectively. Each flask was inoculated with 500 conidia of *B. sorokiniana* and incubated on a rotary shaker (100 rpm) at room temperature (22 °C). Three flasks were harvested from each series at days 0, 1, 2, 4, 7 and 10. The mycelium was separated from the liquid medium by filtering through a pre-weighed 0.45 μm HVLP Millipore filter. The liquid media were transferred to teflon-lined screw-cap test tubes and extracted with 3 × 1 ml of hexane. The three organic extracts were pooled and evaporated to dryness under nitrogen flow. The mycelial weights were determined after freeze-drying the filters overnight. Due to the high tare of the filters and very small amounts of mycelium it was not possible to obtain reliable sample weights on days 1 and 2 in series A and B, or on any of the days in series C. Each filter was transferred to a teflon-lined screw-cap test tube and 1, 2 or 4 ml of methanol plus 91 ng – 91 μg IS (depending on the weight of the mycelium) was added. The ergosterol concentration was also measured in conidia. Seven samples of 50 000–500 000 freeze-dried conidia were weighed and transferred to tubes with methanol and IS.

Variation in ergosterol due to rapid changes of the liquid nutrient concentration

Three series (AC, BA and BC) of conical flasks containing 15 ml/flask of 100% (AC) or 1% (BA and BC)

liquid media, were inoculated with spores as above and incubated on a rotary shaker. At day 4, three flasks from each series were harvested and the mycelium extracted as above. From another three flasks in each series, the liquid medium was discarded and the mycelium in each flask was washed with 5 ml distilled water. To each remaining flask in series AC, 15 ml of 0.1% liquid medium was added and to those in BA and BC, 15 ml of 100% and 0.1% liquid medium, respectively, were added. The flasks were incubated for a further three days on the rotary shaker. At day 7 the remaining three flasks from each series were harvested and treated as above. The different treatments were compared using the Mann-Whitney U-test.

Development of fungal infection in barley leaves

Seeds of barley (cv. Berolina) were sown in 12 cm pots (4 seeds/pot) and placed in a climate chamber with a 16 h photoperiod (36 Sylvania cool white 115 W and 68 bulbs, 50 W) at a photon flux density of 440 mol $m^{-2}s^{-1}$ in the spectral range 400–700 nm. The temperature was 18 °C/15 °C in the light/dark and the relative humidity was 80%. After three weeks the second leaf of nine plants was attached with a rubber band to a plastic rack with the adaxial surface facing upwards. Prior to inoculation the conidia were suspended in 0.5% water agar (Oxoid) with 0.01% Tween 80 (BDH, England). Each leaf was inoculated along the central vein with 5 × 10 μl droplets of the conidial spore suspension (10^4 spores ml^{-1}) resulting in 500 spores/leaf. Leaves inoculated with water agar and Tween 80 served as a control. The plants were incubated in the climate chamber and the leaves harvested 0, 2, 4, 7, and 10 days after inoculation. Disease development in the leaves was scored by visual grading on a scale from 0 (no symptoms) to 5 (severe symptoms). Each leaf was cut and placed in a teflon-lined screw-cap test tube containing 2 ml methanol and 0.91 μg IS. The samples were stored at −20 °C in the dark until extraction of ergosterol was performed.

Test of resistance

Cultivars of the three barley varieties Berolina, SV 88777 and Ca 602202, grown and inoculated as above, were harvested 10 days after inoculation. The leaves were visually graded and then placed in test tubes containing methanol and IS. The samples were stored as above until extraction of ergosterol. The experiments were performed twice.

Extraction of ergosterol from leaves and mycelium

The tubes containing the infected leaf or mycelium, IS and 2 ml methanol were refluxed at 80 °C for 2 h. After cooling the solution was transferred to another tube and the leaf was washed with a further 2 × 0.5 ml of methanol. The pooled methanol extracts were hydrolysed by refluxing with 0.6 ml 4% KOH for 0.5 h at 80 °C. The mixture was cooled and hexane (1.5 ml) and distilled water (1.5 ml) were added to form two liquid phases. The sample was placed in a Vortex mixer for 30 s, centrifuged for 5 min at 4000 rpm, and the organic phase transferred to another tube. The aqueous phase was washed with 1 ml hexane. The two hexane fractions were pooled and evaporated to dryness under nitrogen gas. The same procedure was followed for ergosterol of mycelium alone.

Quantification of ergosterol by HPLC and mass spectrometry (MS)

The chromatography was performed on a Varian Model 5000 HPLC instrument with a Varian Model UV 100 variable wavelength UV detector operating at 282 nm and with a 10 μl sample loop. The column used was a 7.5 cm × 3.9 mm i.d. reverse phase Nova-Pak (C18) (Waters, USA) with a C18 prefilter. The mobile phase was acetonitrile/hexane/2-propanol (90/5/5, V/V/V) at a flow rate of 1 ml min^{-1}. The identity of ergosterol was confirmed by on-line HPLC-MS using a Waters model 600-MS HPLC pump with the column used above and connected to a VG Trio 3 mass spectrometer using discharge assisted thermospray ionisation.

Results

Variation in ergosterol due to fungal age and different nutrient concentration

The mycelial weights as well as the ergosterol contents per flask, increased exponentially over the 10-day experimental period in the A series (100% medium), reaching values of 55.8 mg dw. of mycelium and 155 μg of ergosterol per flask (Figure 1). In the B series (1% medium) the maximum weight and content of ergosterol were reached at day 7 (2.90 mg dw. of mycelium and 11.9 μg of ergosterol per flask); thereafter both

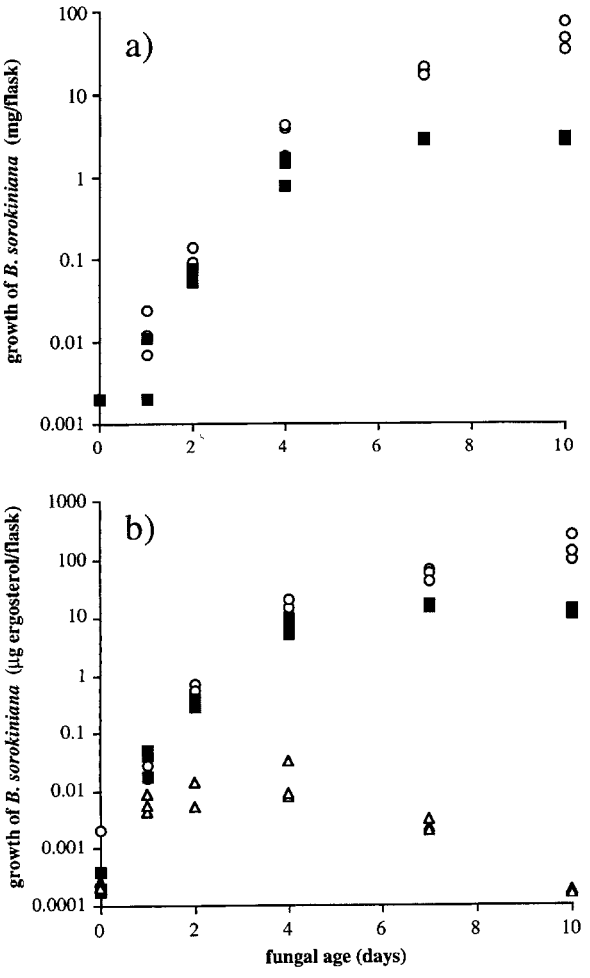

Figure 1. Growth of *B. sorokiniana* in liquid media at different nutrient concentrations (○ 100%, ■ 1% and △ 0.01%) as a function of time. Growth measured by a) weight, b) ergosterol.

the weight and the ergosterol content decreased. Reliable mycelial weights (> 10 µg) could not be obtained in the C series (0.01% medium), whereas the ergosterol content increased until day 4, (0.013 µg per flask) and thereafter declined to almost zero at day 10. The concentration of ergosterol in spores was 0.94 (0.06) µg mg^{-1} [mean (SD)]. The concentrations of ergosterol in mycelium in series A and B decreased with fungal age from day 4 to day 10. It was higher in mycelium from the 1% medium (4–7 µg mg^{-1} dw) than in the 100% medium (2.5–5 µg mg^{-1} dw) (Figure 2). Because of low sample weights, compared to the tare, a correlation could not be made before day 4, or at the lowest nutrient concentration (C-series). The amount of ergosterol released into the liquid medium

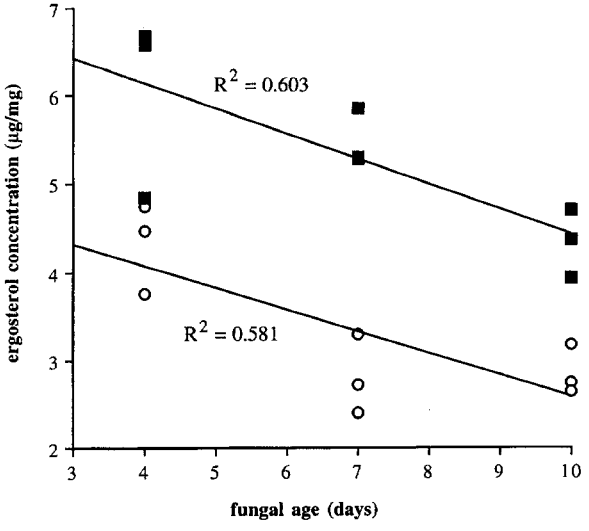

Figure 2. Correlation between ergosterol concentration and fungal age at two different nutrient concentrations (○ 100% and ■ 1%).

was low and could only be determined from day 7 in the A series (11.4 SD 9.9 pg ml^{-1}).

Variation in ergosterol due to rapid changes in the liquid nutrient concentration

The concentrations of ergosterol in the mycelium changed rapidly when the nutrient liquid media were changed (Figure 3). Changing to a less nutrient-rich medium resulted in an increase in the ergosterol concentration (A4 – AC7, $p = 0.0641$; B4 – BC7, $p = 0.0339$) whereas a change to a richer medium led to a decrease (B4 – BA7, $p = 0.0495$). The concentrations of ergosterol varied between 1 and 10 µg mg^{-1} (dw) due to changes in the nutrient medium concentration.

Development of fungal infection in barley leaves (time series)

Signs of infection (necroses) in the leaves were already visible at day 2 when about half of the leaves had a visual infection grading of 1 (light infection). From day 4 to day 10 all plants were visibly infected with mean visual grading from 1.2 to 2.9 (Figure 4). Mass spectrometric analyses of leaf samples confirmed the chromatographic peak as originating from ergosterol. The ergosterol content in the leaves increased exponentially until day 7, thereafter the increase levelled off.

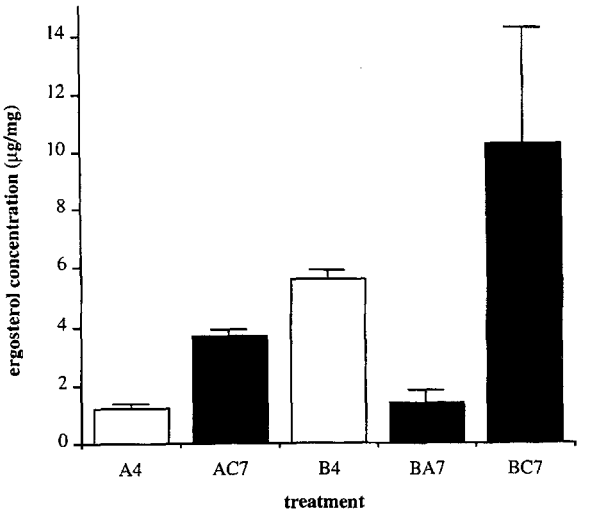

Figure 3. Changes in fungal ergosterol concentrations due to rapid changes in the concentration of nutrient media. A4 and B4; ergosterol concentrations in mycelium after 4 days growth in 100% and 1% liquid media. AC7, BA7 and BC7: ergosterol concentrations in mycelium after 7 days growth but after changing the nutrient liquid media to 0.1%, 100% and 0.1% respectively. See results for statistics.

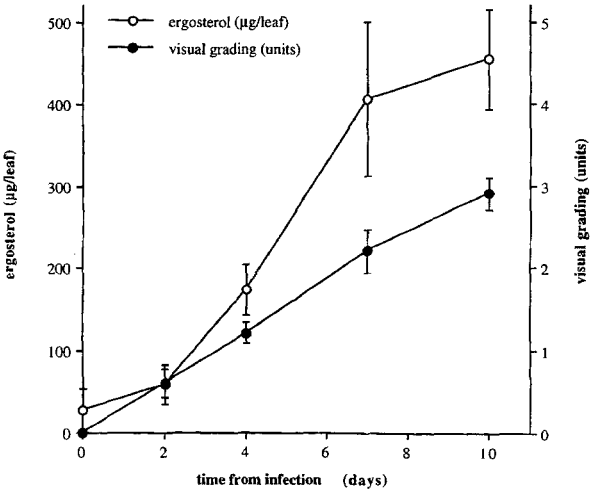

Figure 4. Ergosterol content in leaves and visual grading versus fungal age. Mean of 9 replicates and S.E.

Table 1. Visual grading and ergosterol content in leaves of barley in two repeated experiments (n = 10). Mean and (SD) are given

Barley cultivar	1st experiment		2nd experiment	
	Visual grading	Ergosterol (µg/leaf)	Visual grading	Ergosterol (µg/leaf)
Ca 602202	4.9 (0.4)	10.4 (3.5)	1.9 (0.8)	0.96 (1.3)
Sv 88777	3.8 (0.5)	3.9 (3.0)	1.4 (0.9)	1.01 (0.7)
Berolina	2.4 (0.7)	1.2 (0.9)	1.4 (1.2)	0.57 (1.4)

Figure 5. Three barley cultivars infected with *Bipolaris sorokiniana* after 10 days incubation.

Test of resistance

Both visual grading and measurements of ergosterol content indicated heavier infections in the first experiment compared to the second (Table 1). The cultivar Ca 602022 was visually graded and rated by the ergosterol content as the most susceptible and Berolina as the most resistant in the first experiment. Rating by the ergosterol content in infected leaves was similar to visual grading but was more sensitive in having a greater span with mean values between 0.57 and 10.4 compared to visual grading of 1.4 to 4.9 (Figure 5). The visual grading of the leaf spots was correlated with the content of ergosterol (r = 0.82, n = 24, p = 0.0001, Spearman corr.).

Discussion

The procedure used, involving refluxing and hydrolysation followed by splitting of liquid phases, is rapid (c.f. 16 h at RT, Davis and Lamar, 1992), and compared to the supercritical fluid extraction method (Young and Games, 1993) more efficient. The procedure also reduces the risks of contamination from homogenisation and/or filtration which are often used (e.g. Seitz et al., 1979; Matcham et al., 1985; Nout et al., 1987; Martin et al., 1990). Excluding the filtration step increases the importance of centrifugation after the formation of two liquid phases; otherwise a non-soluble layer, formed between the two liquid phases, seems to increase and the yield of ergosterol decrease. Even for samples rich in small particles (e.g. spores) very little or no solid material was transferred to the HPLC sample tubes indicated by the number of samples that could be run per prefilter on HPLC. Typically 100–200 unfiltered samples could be run, compared to 150–200 samples filtered through the 0.22 μm pore size filter before splitting into two phases. Use of the internal standard indicated a loss of substances of about 15% of both filtered and non-filtered samples during extraction. However, decreasing the time of mixing during extraction to the hexane phase, from 2 × 30 sec to 2 × 15 sec increased losses to about 40%.

The growth curves of *B. sorokiniana* (Figure 1) followed the same pattern either measured as weight or ergosterol changes. The ergosterol measurements are more sensitive than weighing and allow determination of very low amounts of fungus at low nutrient concentrations (0.01%, Figure 1). There was, however, an age-dependent decrease in ergosterol concentration, also found by Gessner and Chauvet (1993) for many aquatic hyphomycetes. This decrease was not a result of leakage of ergosterol to the surroundings; we found that less than 12 pg ml^{-1} was leached to the liquid medium which demonstrates the hydrophobic property of ergosterol. Instead the decrease is probably caused by an increased storage of nutrients in the fungal mycelium, and thus a relatively lower content of ergosterol. If, however, the nutrients are depleted (c.f. Figure 3) the ergosterol concentration may instead increase due to relatively lower nutrient storage. This may explain the increased ergosterol concentration with fungal age found by Torres et al. (1992) and Gessner and Chauvet (1993). Since the spores of *B. sorokiniana* contain less ergosterol than growing hyphae, 0.9 and 4–6 μg mg^{-1} dw respectively, there will probably also be an increasing ergosterol concentration during the early stages of hyphal development.

The differences measured in ergosterol concentration in *B. sorokiniana* (1–10 μg mg^{-1}, Figure 3) have earlier only been reported between different species or strains (Gessner and Chauvet, 1993). This work demonstrates, however, that the fungal ergosterol concentrations are highly dependent on nutrient conditions and that up to ten-fold variations may occur in a few days, or less, in one single fungal strain if the nutrient conditions are changed. This great variation in ergosterol concentration in a single strain limits the use of the method for quantitative analyses, such as the analysis of natural samples. However, in experiments where only one fungal strain is used in a given matrix and inoculation is carried out at the same time, relative quantification may be possible. These criteria can be fulfilled in the resistance rating of different host cultivars. Figure 4 shows that our ergosterol measurements gave a sigmoidal growth curve in the barley leaves which is normal for growth of microorganisms. Visual grading indicated an almost linear growth curve which probably does not reflect the actual development of the infection. Measurement of ergosterol thus often gives a better quantitative assessment of infection than visual grading. Furthermore, in cases where the infections do not visually damage the host plant but give yield decreases, chemical methods such as the ergosterol assay may be the only way to detect an early infection.

Our procedure to measure ergosterol indicated Berolina as the most resistant barley cultivar in both experiments, whereas the visual grading showed this cultivar to be most resistant in the first experiment only. The differences between the two methods may be small, as in this investigation, but due to the objectivity and sensitivity of ergosterol measurements, this method has a great advantage over visual rating.

Conclusion

This work shows that there may be great variations in fungal ergosterol concentrations due to fungal age and nutrient conditions. However, measurements of ergosterol may be used not only to detect fungal infections but also to rate the severity of infection in a given matrix. Extraction of ergosterol from natural samples can often be performed without homogenisation and filtration if refluxing is used. Our procedure was found

to be a useful tool for screening resistance in host plants and was more sensitive and objective than visual grading. it is also more rapid when used on fungus – host combinations that show no visible damage except for host yield losses.

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