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Relationship between production of the phytotoxin prehelminthosporol and virulence in isolates of the plant pathogenic fungus *Bipolaris sorokiniana*

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Abstract

A gas chromatographic method was developed to quantify the phytotoxin prehelminthosporol, which is a sesquiterpene metabolite of the plant pathogen *Bipolaris sorokiniana*. The toxin was extracted from mycelium or culture filtrates, pre-cleaned using solid phase extraction, and analyzed by gas chromatography as a trimethylsilyl-derivative. The detection limit of the method was 5 ng μl^{-1} (signal to noise ratio 4 : 1) which corresponds to ca. 15 ng prehelminthosporol per mg dry weight of mycelium or 15 ng prehelminthosporol per ml culture filtrate. The total amount of prehelminthosporol (mycelium plus culture filtrate) increased with cultivation time when examined in six isolates of *B. sorokiniana* after 6, 9, 12 and 15 days of incubation. The screening experiment of 17 isolates for prehelminthosporol production after 8 days of incubation revealed significant differences in the toxin production between the isolates. The isolates with low toxin production had lower virulence towards barley roots compared to those with higher production of the toxin. However, the virulence did not increase with prehelminthosporol level among the high producing isolates. Prehelminthosporol was also analyzed in a number of related *Bipolaris* and *Drechslera* species. In addition to *B. sorokiniana*, three out of six *Bipolaris* species (*B. setariae*, *B. zeicola*, *B. victoriae*) produced prehelminthosporol, which indicates that ability to produce prehelminthosporol is conserved among closely-related *Bipolaris* species.

Abbreviations: GC – gas chromatography; MS – mass spectrometry; PHL – prehelminthosporol; SPE – solid phase extraction; TMS – trimethylsilyl.

Introduction

Bipolaris sorokiniana is a severe fungal pathogen causing common root rot and leaf spot diseases in wheat, barley, oats and rice, as well as in many other grasses. The pathogen occurs throughout the world (Sivanesan, 1987). However, devastating epidemics are mainly reported from countries with warm and moist climates like India, Thailand, Bangladesh and Brazil (Duveiller et al., 1998). The key factors for the success of the pathogen are not yet discovered and there is still no major resistance available. As a result, disease control depends on an integrated management approach.

Ludwig (1957) presented the first evidence that the pathogenicity of *B. sorokiniana* is related to the production of non-host-specific toxins. The author observed that application of culture filtrates on seedlings of different host plants induced symptoms similar to those of the infecting fungus. Numerous compounds have

since been isolated from cultures of *B. sorokiniana* and the highest phytotoxic activity has been associated with metabolites of sesquiterpene nature (De Mayo et al., 1961; 1965; Pringle, 1976; Pena-Rodriguez et al., 1988; Pena-Rodriguez and Chilton, 1989; Carlson et al., 1991; Nakajima et al., 1994). Among them, prehelminthosporol (PHL) was the most effective in disrupting cell membranes. It was later shown that PHL also affected the activity of enzymes located on the plasma membrane (Carlson et al., 1991; Liljeroth et al., 1994; Olbe et al., 1995). PHL is the major sesquiterpene metabolite produced by *B. sorokiniana* when grown in liquid cultures (Aldridge and Turner, 1970; Cutler et al., 1982; Carlson et al., 1991). The toxin is present in dormant conidia, being released during germination, and is continuously produced during hyphal growth (Carlson et al., 1991).

Although, a number of studies have demonstrated the toxicity of PHL to plants, its role as a virulence factor in *B. sorokiniana* is not clear. In this paper we describe a study on the relationship between the production of PHL and virulence, by comparing toxin production and virulence in a number of different isolates of *B. sorokiniana*. For this reason, a method to isolate and quantify PHL from fungal material was developed.

Materials and methods

Chemicals and solvents

N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) was purchased from Sigma (St. Louis, MO, USA) and pyridine (purity 99.8%) from Fluka (Buchs, Switzerland). 1-Tridecanol (purity 99%) was purchased from Larodan (Malmö, Sweden) and used as an internal standard in gas chromatography (GC) analyses. The solvents used for extractions and chromatography were obtained from Fisons (Loughborough, UK) and were of HPLC grade.

Fungal isolates

Isolates of *Bipolaris sorokiniana* (teleomorph: *Cochliobolus sativus*) were obtained from the following sources: Tellus 1, Tellus 2, Tellus 3, Wellan, and 118 from M. Gustafsson, Department of Crop Genetics and Breeding, The Swedish University of Agricultural Sciences, Svalöv, Sweden; BRD 1, BRA, ZIM 1 and THA 1 from J. Hetzler, Institute für Pflanzenpathologie und Pflanzenschutz, Georg-August Universität, Göttingen-Weende, Germany; 2715, R3-54, and A 18 from K. Bailey, Agriculture Canadas Research Station, Saskatoon, Canada; 510.65 from CBS, Baarn, The Netherlands; K247, R002, K294, and K381 from R.W. Stack, Plant Pathology, North Dakota State University, USA. *Bipolaris victoriae* (329.64), *B. zeicola* (209.79), *B. setariae* (313.64), *B. sacchari* (736.84), *B. oryzae* (310.64), *B. maydis* (573.73), *Drechslera avenae* (307.84), *D. graminea* (314.69) and *D. tritici-repentis* (265.80) were obtained from CBS. *Drechslera teres* (isolate nr 8 from cv. Tellus) was a gift from Svalöv-Weibull AB, Sweden.

Cultivation of fungi

All fungal isolates were maintained on agar slants or as freeze-dried conidia at 4 °C. For production of inoculum, the fungi were grown on a defined agar medium (Skoropad and Arny, 1957) at room temperature (24 °C) for 9–18 days. In the experiments for PHL production, 100 ml of a defined liquid medium was inoculated either with 6×10^5 conidia (isolates of *B. sorokiniana*) collected as previously described (Carlson et al., 1991), or with agar plugs (*B. victoriae*, *B. zeicola*, *B. setariae*, *B. sacchari*, *B. oryzae*, *B. maydis*, *D. avenae*, *D. graminea*, and *D. tritici-repentis*). The fungi (triplicates of each fungus) were grown on a rotary shaker (150 rpm) at room temperature (25 °C) and harvested after 8 days. In addition, the isolates K247, BRD 1, THA 1, Tellus 1, K381 and R3-54 were cultivated for 6, 9, 12 and 15 days at 20 °C. The mycelium was separated from the culture liquid by vacuum filtration through two sheets of filter paper. The mycelium and the culture filtrates were stored at −20 °C.

Extraction of prehelminthosporol

PHL was extracted from the culture filtrates using diethyl-ether (Carlson et al., 1991). Briefly, three volumes of culture filtrate were shaken twice with one volume of diethyl-ether. The combined ether extracts were washed with 1.8 volume of distilled water and evaporated under N_2. The extracts were further dried in a lyophilizer (overnight), and thereafter stored at −18 °C. The mycelium was freeze-dried and homogenized using a mortar and pestle. PHL was extracted from the mycelium (approximately 100 mg dry weight) using the method of Bligh and Dyer (1959), as modified by Carlson et al. (1991). The extracts were stored at −18 °C.

Pre-cleaning of prehelminthosporol

The extracts were dissolved in hexane–ethylacetate (9 : 1, v/v). An aliquot of 30 µl (typically containing between 20 and 200 ng PHL per µl) of the crude extract was applied onto a solid phase extraction (SPE) column (CG, Bond-Elut containing 100 mg silica gel, Analytichem, Harbour City, CA, USA). The cartridges were pre-conditioned with 1 ml of hexane and equilibrated with 1 ml of hexane–ethylacetate (9 : 1, v/v) before application of the samples. The sample was eluted with 1.7 ml of hexane–ethylacetate (9 : 1, v/v) and collected in a test tube spiked with 30 µl (46 ng μl^{-1}) of the internal standard (1-tridecanol, in hexane). The solvent was evaporated with N_2 (room temperature). To test the recovery of PHL in the SPE cleaning procedure, crude extracts of 8-day-old Tellus 1 cultures were divided in two series (each with three replicates), and one of the series was spiked with 200 ng μl^{-1} of PHL. Both series were purified using the SPE method, derivatized and analyzed by GC.

Derivatization and gas chromatography

The samples were silylated with 10 µl of BSTFA in 20 µl dry pyridine and 1 µl of the reaction mixture was directly injected on the GC. A Hewlett Packard 5890 series II gas chromatograph (in the time course study) or a Varian model 3700 gas chromatograph (in the screening experiment) equipped with a flame ionization detector (FID) and a fused silica DB-5 (film thickness 0.25 mm) capillary column (30 m × 0.25 mm ID) were used. The temperature of the injector was 250 °C and of the detector 300 °C. The column temperature was initially held at 130 °C for 3 min and thereafter increased by 3 °C min^{-1} to 220 °C. Injections were made in the splitless mode (injection volume 1 µl). The flow of the carrier gas (hydrogen) was 1.0 ml min^{-1}.

Quantifications

A standard of PHL was obtained by extracting the toxin from *B. sorokoniana* (Carlsson et al., 1991). The concentration of PHL in the standard was determined by assuming that the response factor of PHL was identical to that of the internal standard (1-tridecanol). A calibration curve was constructed by adding various amounts of the PHL (11–184 ng μl^{-1}) to 1-tridecanol (46 ng μl^{-1} in hexane), followed by trimethylsilyl (TMS)-derivatization and GC/FID analysis. The detection limit of the method was determined by diluting the PHL standard (in hexane–ethylacetate, 9 : 1), followed by TMS-derivatization and GC/FID analysis. In all analyses using the TMS derivatives, PHL was quantified by relating the peak heights of both epimers to that of the internal standard.

Mass spectrometry

Mass spectrometry (MS) was performed using a VG Trio-1S MS instrument connected to a Hewlett Packard 5890 GC with a DB-5 column. Helium was used as the carrier gas at a flow rate of 1.0 ml min^{-1}. GC conditions were similar to those described above. Electron impact (EI) mass spectra were obtained using 70 eV ionizing energy and an ion source temperature of 220 °C.

Screening of virulence

The ability to produce disease symptoms on roots of young barley seedlings was assessed, using a method described by Liljeroth et al. (1993). Briefly, seeds of the susceptible barley variety Harry (W. Weibull AB, Landskrona, Sweden) were sterilized and germinated on moist filter paper for 24 h. The Harry variety was selected because previous studies have shown that it is sensitive to PHL (Liljeroth et al., 1994). Seeds with emerging roots were transferred to filter paper sheets. Another paper sheet with a plastic layer was placed on top, and the resulting sandwich was rolled together, with the lower end of the roll placed in distilled water. *Bipolaris sorokiniana* inoculum was prepared by adding 10^5 spores to 10 ml of Czapek Dox agar (Oxoid) in a Petri dish. After 2 days at 25 °C, 5-mm agar disks were cut with a cork borer and used as inoculum on 8-day-old seedlings. The fungal inocula were placed on the roots 5 cm below the seed. Two rolls (15–20 plants) were used for each fungal strain. The extent of disease symptoms, indicated by a brown discoloration, was measured as millimeters of infected root 7 days after inoculation. When screened, the isolates were divided in three groups, and in each group the isolate Tellus 1 was included. The extent of disease symptoms for each isolate was compared with the symptoms caused by the Tellus 1 isolate. The experiment was repeated twice.

Statistical analyses

Statistical analyses were performed using one-way ANOVA and Student's *t*-test.

Results

GC quantification of prehelminthosporol

Initial analysis showed that the extracts of the culture filtrate and the mycelium contained substances having GC retention times similar to that of PHL. The interfering compounds were removed by including a cleaning step using a SPE column. The recovery of PHL using this extraction procedure was 97 ± 3% (mean ± SD, $N = 3$). TMS-derivatization of PHL resulted in two peaks in the GC chromatogram (Figure 1). The mass spectra of the two O-TMS peaks of PHL were identical (Figure 2). Both spectra had weak molecular ions of m/z 308. The peak of m/z 293 (M-15) is due to cleavage of the methyl to silicon bond; m/z 73 represents $(CH_3)_3Si^+$, and m/z 147 represents $(CH_3)_2Si{=}^+OSi(CH_3)_3$ ions. Due to the fact that the MS spectra of the two PHL peaks were identical, they most probably represent the epimers at the hemiacetal carbon atom.

The calibration curve showed good linearity within the concentration range tested (Figure 3). The detection limit of the method was approximately 5 ng PHL per µl (signal to noise ratio 4 : 1). Assuming an extraction of 100 ml of filtrate or 100 mg of mycelium, the obtained detection limit corresponds to 15 ng PHL per ml culture filtrate and 15 ng PHL per mg dry weight mycelium, respectively.

PHL production by Bipolaris sorokiniana

Initially the production of PHL in six isolates (R3-54, K381, Tellus 1, BRD 1, THA 1, K247) of *B. sorokiniana* was analyzed. In all isolates, PHL production increased with cultivation time, despite differences in growth patterns (Figure 4). For example, the isolates Tellus 1 and BRD 1 reached the stationary phase after 9 days of incubation, while the isolate K247 was still in a growth phase after 15 days (data not shown).

For a screening of PHL production in a larger number of isolates, 17 isolates at one sampling time (8 days of incubation) were used. The intention was to measure PHL production before the fungi reached the stationary growth phase because at the latter a breakdown of

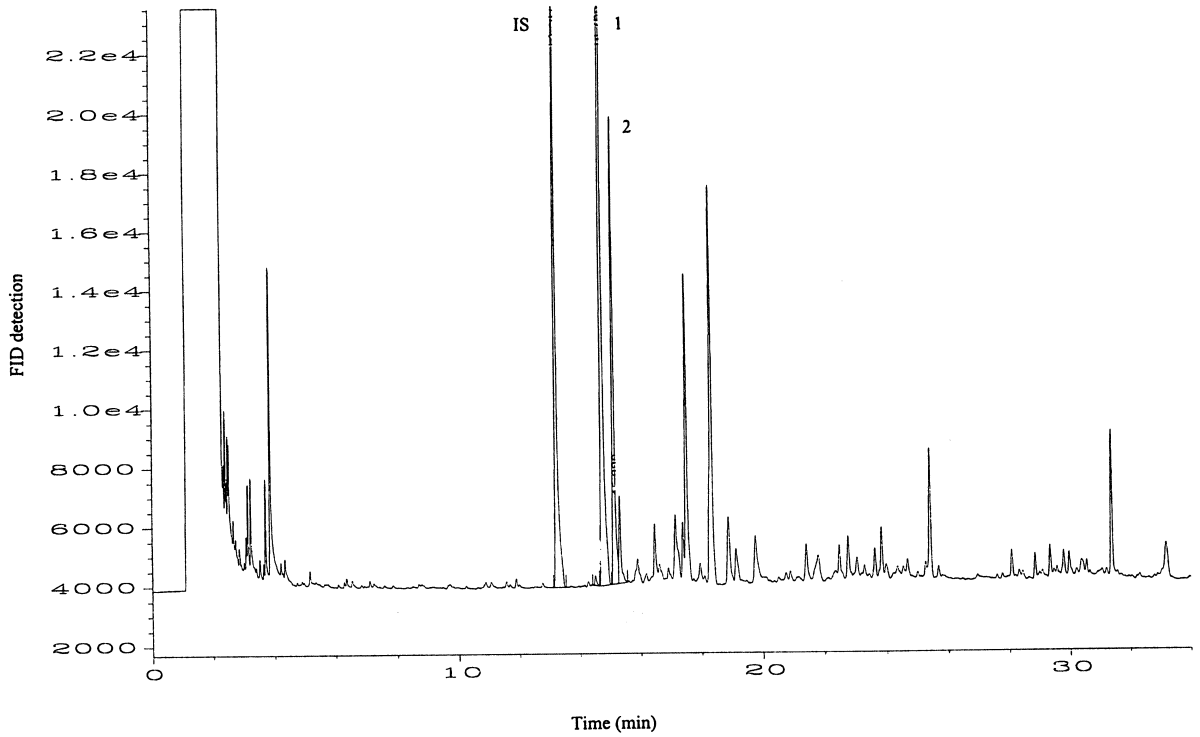

Figure 1. Gas chromatogram of PHL (peaks 1 and 2) extracted from culture filtrate of K381, pre-cleaned on SPE column and TMS derivatized. IS = internal standard.

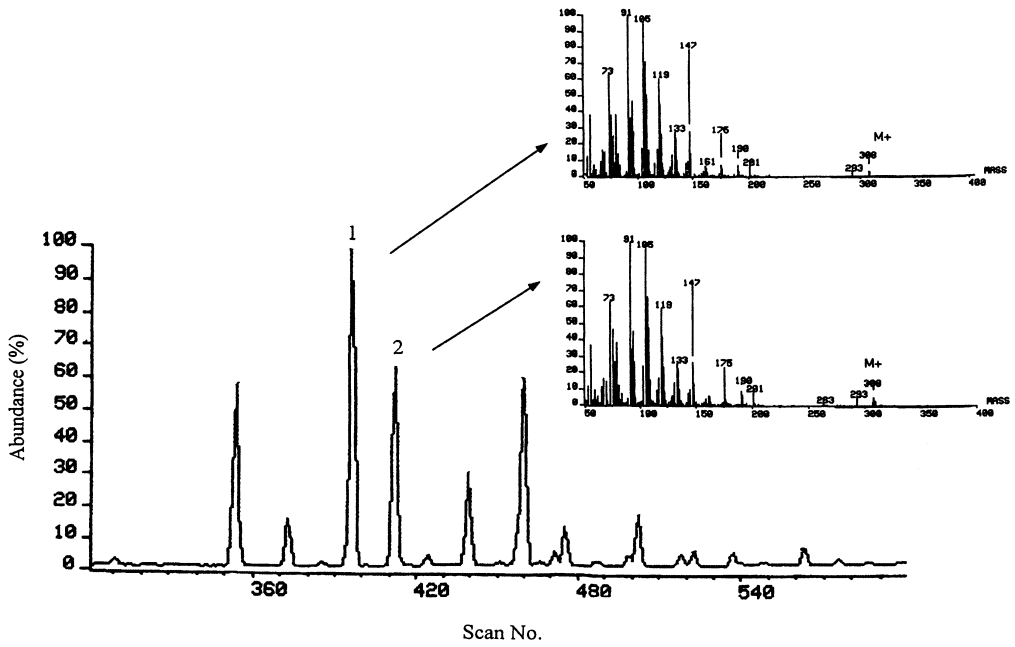

Figure 2. Total ion chromatogram and mass spectra (inserts) of O-TMS PHL epimers 1 and 2 extracted from culture filtrate of BRD III. M^+ = molecular ion.

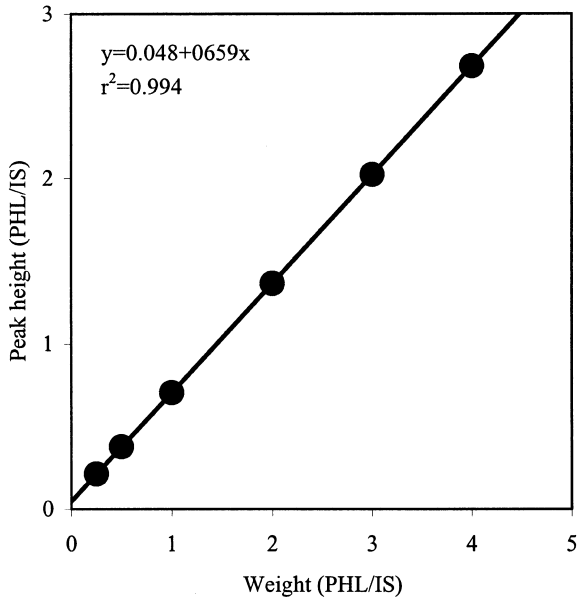

Figure 3. Calibration graph for the TMS derivative of PHL. IS = TMS derivative of the internal standard (1-tridecanol).

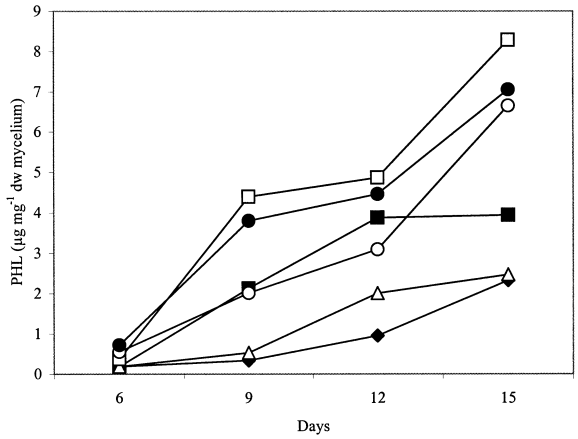

Figure 4. Total amount of PHL (mycelium and culture medium) produced by isolates R3-54 (♦), K381 (■), Tellus 1 (Δ), BRD 1 (□), THA (◯) and K247 (●) at 22 °C. Values represent means of three replicates. The average SE was 14% of the mean values.

the toxin may occur (Nilsson et al., 1993). The total amount of PHL (mycelium plus culture filtrate) differed significantly between the 17 analyzed isolates of *B. sorokiniana* (one way ANOVA test, $P < 0.001$). The amount of PHL produced was within the range of 0.6–4.4 µg mg^{-1} dry fungal tissue. Thus, PHL production differed between the lowest (isolate R3-54) and the highest producers (K247) by a factor of 6. In most samples, the quantity of PHL in the culture medium and the mycelium was similar.

Virulence of Bipolaris sorokiniana

The virulence differed significantly between the various isolates of *B. sorokiniana* (Table 1). The most virulent isolates in this test were Welan and R002, whereas R3-54 and 510.65 had lowest virulence. When the experiment was repeated, the same order of virulence was observed, despite somewhat different virulence indices.

Relationship between PHL production and virulence

There was a significant curvelinear relationship ($y = a + b/x$; $P = 0.029$) between the amount of PHL produced and the virulence of isolates of *B. sorokiniana*, indicating that isolates with low toxin production had lower virulence compared to those with higher production of toxin. However, the virulence did not increase with PHL level among the high toxin-producing isolates (Figure 5).

Table 1. Virulence of various isolates of *Bipolaris sorokiniana*. Disease symptoms were assayed by comparing the size of lesions on barley roots (mean ± SE (N))[a]

Isolate	Lesion size (mm)	Virulence index
R002	38 ± 1.2 (19)***	1.31
K247	34 ± 1.7 (10)*	1.18
BRD 1	33 ± 1.0 (19)*	1.14
THA 1	31 ± 1.4 (20) ns	1.07
K381	24 ± 1.2 (20)*	0.84
R3-54	17 ± 1.2 (16)***	0.58
Tellus 1	29 ± 1.2 (17)	1.00
Welan	39 ± 1.2 (13)***	1.33
Tellus 3	37 ± 1.0 (17)***	1.27
K294	35 ± 1.6 (16)*	1.21
2715	29 ± 0.8 (18) ns	1.00
A18	19 ± 1.5 (19)***	0.66
510.65	17 ± 1.3 (15)***	0.57
Tellus 1	29 ± 1.7 (15)	1.00
118	32 ± 1.2 (15)*	1.14
Tellus 2	29 ± 0.9 (19) ns	1.05
BRA 111	28 ± 1.2 (19) ns	0.99
ZIM 1	24 ± 0.9 (15)*	0.87
Tellus 1	28 ± 1.2 (19)	1.00

[a]The inoculum was placed on roots of 8-day-old barley seedlings. Lesion size was measured 7 days after inoculation. The Tellus 1 isolate was included as a reference in each of the 3 groups of isolates tested. The significance of differences between each isolate and the Tellus 1 isolate was investigated with Student's t test. ns: not significantly different from the control, *: $P < 0.05$, ***: $P < 0.001$. Virulence indices were calculated by dividing the mean of each isolate with the mean of the Tellus 1 isolate.

PHL production in Bipolaris *and* Drechslera *species*

Screening of PHL in different *Bipolaris* and *Drechslera* species showed that the toxin was present in three species (Table 2). In addition to isolates of *B. sorokiniana*, *B. victoriae*, *B. zeicola* and *B. setariae* were also PHL producers. Under the conditions used in this study, *B. victoriae* produced the smallest amount of PHL (0.10 μg mg^{-1} dry weight of mycelium).

Discussion

For more than 40 years, it has been assumed that non-host-specific toxins like PHL are responsible for the disease severity caused by *B. sorokiniana* (Ludwig, 1957; Kurppa, 1985; Hodges and Campbell, 1999).

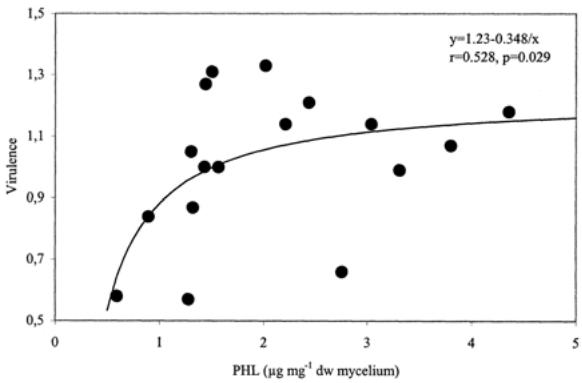

Figure 5. Relationship between PHL production (8 days of incubation, 25 °C) and virulence of isolates of *Bipolaris sorokiniana*.

Table 2. Amount of PHL produced by species related to *Bipolaris sorokiniana* after 8 days of cultivation. Mean ± SD ($N = 3$)

Species	PHL (μg mg^{-1} dw mycelium)
B. setariae	2.2 ± 0.9
B. zeicola	1.6 ± 0.2
B. victoriae	0.1 ± 0.1
B. maydis	ND[a]
B. oryzae	ND
B. sacchari	ND
D. avenae	ND
D. graminea	ND
D. teres	ND
D. tritici-repentis	ND

[a]ND, not detected (<0.03 μg mg^{-1} dw mycelium).

This suggestion has been based on the fact that culture filtrates, when applied to host plants, reproduced symptoms similar to those induced by the fungus itself. In this paper, we present evidence that a correlation exists between *in vitro* PHL production and the degree of virulence of isolates of *B. sorokiniana* towards barley seedling roots. The isolates with low PHL production had lower virulence compared to those with higher production of PHL. However, virulence did not increase with PHL level among the high toxin-producing isolates. The reason for this is not known; possibly the high concentration of PHL elicits host defence reactions that restrict further disease development.

Several methods have been developed for the analysis of PHL. Carlsson et al. (1991) used HPLC/MS. Although the sensitivity of this method was similar to that of the GC method described in this paper, it has limited use in routine screening experiments since an expensive MS detection system is needed. Nilsson et al. (1993) analyzed non-derivatized PHL using GC. The advantages of using TMS derivatives are: (1) higher sensitivity due to sharper GC peaks compared to the peak of underivatized PHL; the slope coefficients of the dose-response curves for the TMS-derivatized PHL was 27% higher than that for non-derivatized PHL (Apoga, unpublished data); (2) easier identification of the PHL peaks in the GC chromatogram; the dual peaks of the TMS-derivatized PHL had characteristic retention times and distance between the signals, which facilitated identification of PHL in the GC chromatograms. Furthermore, the introduction of a fast, pre-cleaning step using SPE in the presented method was important for removing interfering contaminants in the samples.

Screening of PHL production of 17 isolates of *B. sorokiniana* from different geographical origins showed that all the isolates produced the phytotoxin, but at different levels and rates. To establish a relationship between the production of PHL and virulence, toxin production was measured during *in vitro* growth in liquid cultures. To further confirm the involvement of PHL during pathogenesis, the production of PHL *in vivo* and its relationship to disease symptoms should be analyzed. However, the present method for assaying PHL does not have the sensitivity for such measurements. Localization of the toxin at the infection site using antibodies and microscopy techniques could be important for demonstrating the function of PHL in the host plant. Polyclonal antibodies against PHL have been produced and successfully applied to discover PHL localization in Woronin bodies within the fungus (Åkesson et al., 1996).

Another strategy to establish the role of PHL during infection would be to generate mutants of *B. sorokiniana* lacking or being deficient in producing PHL. Targeted gene disruption has been a very successful approach for analyzing the function of host-specific toxins in the genus *Cochliobolus* (Yoder et al., 1997). However, the genes involved in the biosynthesis of PHL have not yet been identified. Furthermore, *B. sorokiniana* contains a number of sesquiterpene metabolites that are structurally related to PHL (De Mayo et al., 1961, Sommereyns and Closset, 1978; Carlson et al., 1991; Briquet et al., 1998). Thus, it can be expected that the phytotoxicity of the fungus probably depends on the synergistic action of several phytotoxins. A correlation between victoxinine (a minor phytotoxin of *B. sorokiniana*) production and aggressiveness has also been reported (Pringle, 1977).

Three out of six *Bipolaris* species (*B. setariae*, *B. zeicola*, *B. victoriae*) in addition to *B. sorokiniana* produced PHL, showing that production of this toxin is conserved among closely-related *Bipolaris* species. Scheffer (1991) proposed that *B. sorokiniana*, which has a wide host range (non-host-specific toxin producer) is an ancestral species to specialized pathogens like *B. maydis*, *B. victoriae* and *B. zeicola* (host-specific toxin producers), and that the major difference among these species is due to the presence of genes responsible for host-specific toxin production. It has previously been shown that *B. sorokiniana* (*C. sativus*), *B. zeicola* (*C. carbonum*) and *B. victoriae* (*C. victoriae*) are so closely related that interfertile sexual crosses were possible (Nelson, 1960; Scheffer, 1991). Recent phylogenetic analysis of *Bipolaris* using the *gpd* (glyceraldehyde-3-phosphate dehydrogenase) and the *Brn1* (a reductase gene involved in melanin synthesis) gene sequences has confirmed the close relationship among *Bipolaris* species (Berbee et al., 1999; Shimizu et al., 1998). However, the earlier hypothesis that *B. sorokiniana* is an ancestor to the specialized species, has not been supported by these studies. Analyses of more variable gene sequences are needed to reveal the evolutionary relationships between toxin and non-toxin producing species of *Bipolaris*.

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