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Virulence–Toxin Production Relationship in Isolates of the Plant Pathogenic Fungus *Botrytis cinerea*

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Abstract

Eleven isolates of *Botrytis cinerea* were studied to examine the relationship between toxin production and virulence. After 5 days of incubation, screening experiments revealed significant differences in toxin production by the strains. The isolates with low toxin production were less virulent; moreover, the only toxins isolated were those corresponding to botrydial or its derivatives. In contrast, higher amounts of toxins were isolated from the more aggressive isolates. Furthermore, two classes of toxins, those with botryane skeleton and botcinolide derivatives, were detected in and isolated from all aggressive strains studied. This indicates that a synergistic action of several toxins is involved in the phytotoxicity of this phytopathogen.

Introduction

The ascomycete *Botrytis cinerea* Pers.:Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetzel] is a phytopathogenic fungus that grows as a grey mould on a variety of commercial crops causing serious economic losses (Verhoeff et al., 1992). A number of phytotoxins have been isolated from this fungus (Collado et al., 2000), the best known being botrydial (**1**) and dihydrobotrydial (**2**), both of which possess the sesquiterpenoid botryane skeleton (Fehlhaber et al., 1974). These metabolites are responsible for the typical lesions of the fungal infection and they play an important role in the pathogenicity of the organism *in vivo* (Colmenares et al., 2002). A second family of toxins with a polihydroxilated lactone skeleton (Fig. 1) has also been reported (Cutler et al., 1993, 1996; Jacyno et al., 1994; Collado et al., 1996).

Despite several decades of research, the mechanism by which *B. cinerea* induces host cell death, the initial event of the necrotrophic life cycle, is still not yet well understood (Prins et al., 2000; Colmenares et al., 2002). Cell wall-degrading enzymes (ten Have et al., 1998), phytotoxins (Rebordinos et al., 1996; Collado et al., 2000), active oxygen species (von Tiedemann, 1997; Liu et al., 1998) or membrane transporters for secretion of plant defence compounds (Schoonbeek et al., 2001) have all been implicated as pathogenicity factors.

Without excluding other factors, circumstantial evidence for a putative role for botrydial (**1**) and its derivatives in the pathogenicity of the organism *in vivo* (Rebordinos et al., 1996; Colmenares et al., 2002) has been reported, along with evidence that the phytotoxin botrydial (**1**) has an action mechanism that is light-dependent. Furthermore, exogenous applications of **1** have been found to facilitate fungal penetration and colonization of plant tissue (Colmenares et al., 2002).

These results, together with the recent discovery that botrydial (**1**) occurs in infected plants (Deighton et al., 2001), suggest the involvement of this compound as a toxin in the pathogenesis by *B. cinerea* . Nevertheless, a correlation between production of toxins and pathogenicity for this fungus has yet to be made.

We describe here the relationship between the production of toxins and virulence in a number of different wild isolates of *B. cinerea*.

Materials and Methods

General experimental procedures

^{1}H and ^{13}C-NMR spectra were recorded on Varian Gemini (Varian Inc., Palo Alto, California, USA) 200 MHz (^{1}H at 199.975 MHz, ^{13}C at 50.289 MHz) and Varian Unity (Varian Inc., Palo Alto, California, USA) 400 MHz (^{1}H at 399.952 MHz, ^{13}C at 100.570 MHz) spectrometers. Chemical shifts are quoted relative to TMS (Me_4Si) in $CDCl_3$. Mass spectra were recorded on a GC–MS Thermoquest (Thermoelectron Corporation, San Jose, California, USA) spectrometer, model Voyager. HPLC was performed with a Hitachi/Merck L6270 (Merck KGaA, Darmstadt, Germany) apparatus

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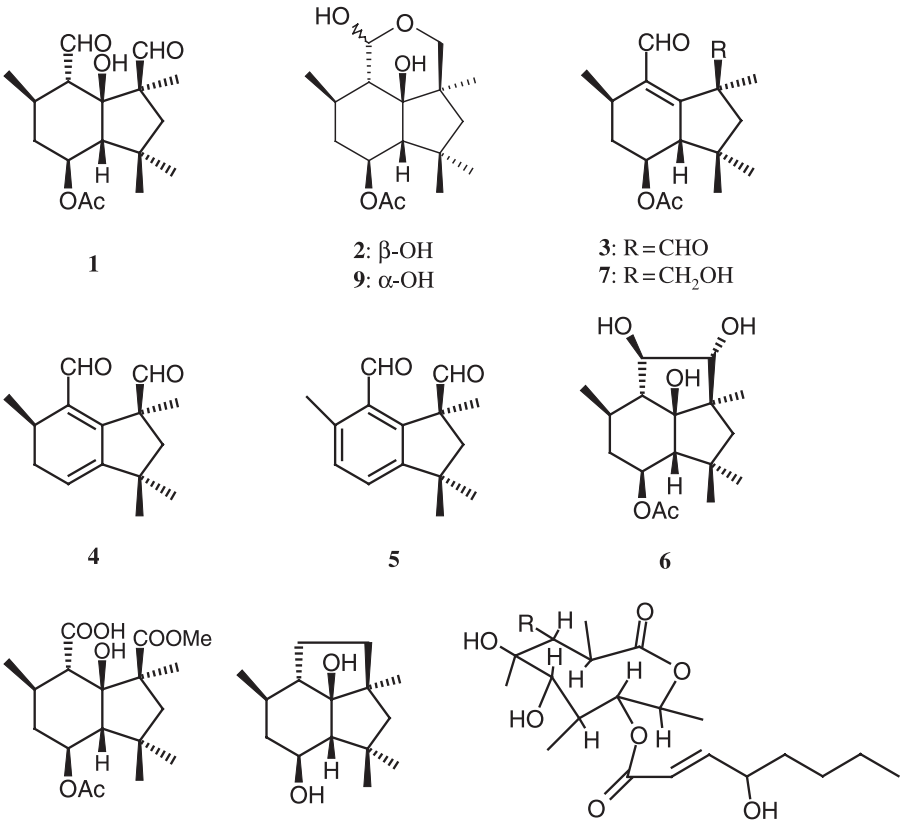

Fig. 1 Structure of compounds from *Botrytis cinerea* strains

equipped with a UV-VIS detector (L4250) and a differential refractometer detector (RI-71). TLC was performed on Merck Kiesegel 60 F_{254}, 0.25 mm thick. Silica gel (Merck) was used for column chromatography. Purification by means of HPLC was carried out with a Si gel column (LiChrospher Si-60 (Merck KGaA, Darmstadt, Germany), 10 μm, 1 cm wide, 25 cm long or 5 μm, 0.4 cm wide, 25 cm long).

Fungal isolates

The place and host of origin, and the relative aggressiveness on bean leaf discs of the 11 strains of the phytopathogenic fungus *B. cinerea* that were studied are shown in Table 1.

Culture conditions

In order to determine the best culture conditions for production of toxins, isolates B1.29 and B1.30 were grown in 18 flasks (500 ml) each containing 200 ml of Czapek–Dox medium (Durán-Patrón et al., 2000). The pH of the medium was adjusted to 7.0. Each flask was inoculated with 2×10^7 fresh conidia and then incubated at 24–26°C on an orbital shaker at 0.9 g. The mycelium from two flasks was separately removed by centrifuging the cultures for 5–10 min on days 1–8 and 13, and the broth was extracted as described below. The toxins botrydial (**1**), botcinolide (**12**) and their derivatives were purified and quantified.

Using the conditions optimised in these pilot experiments, the 11 strains of *B. cinerea* were grown separately in 20 flasks under the conditions described above and incubated for 5 days (10 flasks) or 8 days (10 flasks), at which time all isolates had reached the stationary phase.

Extraction, detection, and isolation of toxins

The broth (2 l) was saturated with NaCl and extracted with EtOAc. The EtOAc extract was washed with H_2O

Table 1
Relative aggressiveness, laboratory and host of origin of various isolates of *Botrytis cinerea*

Isolate code	Place of origin	Original host plant	Relative aggressiveness (%)
B1.11	University of Rostock	*Vitis vinifera*	0
B1.20	University of Rostock	*Hordeum vulgare*	100
B1.26	University of Münster	Selected by P. Büttner	80
B1.29	University of Cádiz	Standard Collection, Coleccion Española Cultivos Tipo	70
B1.30	University of Cádiz	*Vitis vinifera*	94
B1.34	Volcani Center	Tomato	96
B1.35	Volcani Center	Pepper	92
B1.36	Volcani Center	Eggplant	40
Bc I-14	Volcani Center		90
Bc I-21	Volcani Center		35
Bc I-35	Volcani Center		70

and then dried over anhydrous Na_2SO_4. Evaporation of the solvent at reduced pressure gave a yellow oil that was separated by means of column chromatography on silica gel, with mixtures of ethyl acetate/petroleum ether (30, 60 and 100% ethyl acetate) as solvent. Three fractions were collected and labelled as A, B, and C. These were then analysed by means of GC–MS with the authentic sample addition method. Samples of the metabolites **1**–**12**, previously isolated from strains B1.29 and B1.30 (Collado et al., 2000), were used as references in the analysis.

In addition, compounds eluted in fractions A–C were further purified by means of semi-preparative HPLC (hexane : ethyl acetate 90 : 10; 2.8 ml/min) and quantified (Table 2).

Virulence screening

Inoculation experiments

Sporulating fungal colonies of the *B. cinerea* isolates were first rinsed with an inoculating medium consisting of 3.2 g/l Gamborg's B5 solution (Sigma Chemical Company, St. Louis, Missouri, USA), 10 mM sucrose and 0.01 M potassium phosphate buffer at pH 6.4, and then filtered through cheesecloth to eliminate mycelial fragments. The conidial suspension was centrifuged, resuspended in an inoculating medium containing sucrose, and adjusted to approximately 10^5 conidia/ml. Drops (five per leaflet) containing *B. cinerea* conidia were placed on detached *Phaseolus vulgaris* leaflets.

Disease severity was determined on detached leaflets of *P. vulgaris* after 6 days, using the method described by Guetsky et al. (2002) with minor modifications. Under these controlled conditions, the diameter of the drops was identical in each case and the total surface area of the drops was considered to be the treated area. The lesion diameter of aggressive *B. cinerea* strains formed within 6 days was approximately 8–10 mm. A 10-mm-diameter lesion was used as a base size and assigned a value of 100% (Guetsky et al., 2002). Aggressiveness was calculated as the percentage of diseased leaf area expressed as a mean value of 15 leaf discs. The lesion size was scored 3 days postinfection. The most aggressive isolate, B1.2, with a 10 mm diameter lesion, was set to 100% whereas the least aggressive isolate, B1.11, was set to 0%.

Results

Eleven isolates of *B. cinerea* from different geographical origins and of various degrees of aggressiveness were selected for the study (Table 1).

In order to determine the time frame for the production of botrydial (**1**), botcinolide (**12**), and their derivatives, isolates B1.29 and B1.30 were fermented and the toxin production was measured during the *in vitro* growth in liquid culture. The mycelium from two flasks was filtered separately on days 1–8 and day 13 and the broth was extracted with ethyl acetate. The metabolites **1**–**3** and **12** were separated by means of HPLC and their structures determined after extensive mono- and bi-dimensional NMR experiments.

The results showed that botrydial (**1**) and botryenedial (**3**) production started on the second day of fermentation, reaching a maximum on day 5. Botcinolide (**12**) exhibited a similar production pattern, reaching a maximum between days 5 and 6. Dihydrobotrydial (**2**) reached a maximum on day 13, showing a progressive accumulation throughout the fermentation.

To establish a relationship between toxin production and virulence in the 11 wild *B. cinerea* isolates, the toxins were measured for all strains during *in vitro* growth in liquid culture. The isolates were incubated for either 5 or 8 days. The differences between these experiments were not significant.

After filtration and extraction, the broth from all isolates was initially studied with the aid of TLC and GC–MS. However, the presence of metabolites with structures and retention times in GC similar to those of botrydial (**1**) (Collado et al., 2000) resulted in an unclear analysis and no reproducible results. For this reason, the metabolites from all the strains studied were isolated by means of column chromatography and HPLC and their weights determined. Extensive spectroscopic methods were then employed to study the structures of the toxins, which were compared with authentic samples.

All isolates produced phytotoxins, although at different levels and rates (Table 2). The most aggressive isolates (relative aggressiveness >90) produced botcinolide (**12**) or some derivative of this polyhydroxylated lactone. Assuming that botrydial is metabolised by the fungus to the less toxic compounds **2**–**4**, **7** and **8** and that the aggressiveness (on *P. vulgaris*) of each

Table 2
Amounts of toxins produced by isolates of *Botrytis cinerea*

Isolate code	Isolated metabolites* (mg)	Relative aggressiveness (%)
B1.11	**3** (3.0)	0
B1.20	**3** (5.1), **4** (3.5), **12** (5.3)	100
B1.26	**1** (10.1), **2** (6), **3** (5), **6** (12)	80
B1.29	**1** (25.7), **2** (8.5), **3** (2.3), **4** (2.2), **6** (12.0), **7** (6.1), **8** (1.6)	70
B1.30	**1** (5.0), **2** (75.0), **3** (20.3), **4** (20.2), **6** (80.0), **11** (6.0), **12** (3.0)	94
B1.34	**2** (12.8), **3** (21.0), **4** (20.0), **5** (3.3), **6** (31.6), **7** (13.9), **9** (5.5), **8** (22.0), **11** (5.2)	96
B1.35	**10** (15.0), **11** (5.6), **12** (10.1)	92
B1.36	**3** (1.3), **4** (1.5), **8** (7)	40
Bc I-14	**3** (25.1), **4** (10.2), **6** (31.0), **8** (15.1), **9** (5), **11** (1.5)	90
Bc I-21	**3** (2.1), **4** (1.8), **6** (3.8)	35
Bc I-35	**3** (15.0), **4** (10.1), **6** (17.4), **8** (5.5)	70

*Bold numbers refer to compounds illustrated in Fig. 1.

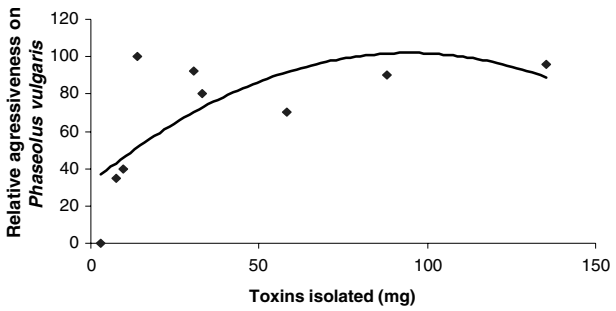

Fig. 2 Relationship between toxin production and relative aggressiveness of isolates of *Botrytis cinerea*

individual strain was related to those of strains B1.20 and B1.11, there was a good correlation between the production of toxins and virulence.

There was a significant curvelinear relationship (Fig. 2) between the total amount of toxins (botryane and botcinolides) and the relative aggressiveness of isolates of *B. cinerea*, indicating that isolates with low botryane toxin production were less virulent than those with higher production of such toxins. Isolates producing botcinolides or its derivatives displayed a much higher degree of aggressiveness.

Discussion

It has recently been reported that exogenous applications of the phytotoxin botrydial (**1**) induces severe chlorosis and cell collapse, thereby facilitating fungal penetration and colonization of plant tissue. The results showed a light-dependent action mechanism for the phytotoxin and seemed to indicate that at least botrydial is a non-host specific toxin, which is involved, in fungal infection (Colmenares et al., 2002).

Our results show that a correlation exists between *in vitro* production of botrydial (**1**), botcinolide (**12**) and their derivative toxins and the degree of virulence in the 11 *B. cinerea* strains. The isolates with low botryane toxin production were less virulent than those with higher production of toxins.

The most aggressive isolates biosynthesize botcinolide (**12**) or one of its derivatives. This new and noteworthy finding suggests a synergistic action of several toxins in the phytotoxicity of this phytopathogen.

To confirm the involvement of the toxins described herein during pathogenesis, the production of botrydial (**1**) and botcinolide (**12**) *in vivo* and its relationship to disease symptoms should be analysed. Recently, botrydial (**1**) was detected for the first time in plant tissues infected with isolates of *B. cinerea* (Deighton et al., 2001). However, the interferences produced by the botrydial derivatives along with the quick biotransformation of **1** to other derivatives limited the utility of the reported procedure. Localization of the toxins at the infection site using antibodies and microscopy techniques may thus be important for demonstrating the function of toxins **1** and **12** in the host plant.

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