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THE PHYTOTOXIC ACTIVITY OF SOME METABOLITES OF *BOTRYTIS CINEREA*

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Key Word Index—*Botrytis cinerea*; Hyphomycetes; fungal metabolites; phytoxicity; botrydial; dihydrobotrydial.

Abstract—A fungus-free culture filtrate from a static culture of *Botrytis cinerea* reproduced the symptoms of the 'grey mould' disease on tobacco leaves. This aspect of the phytoxicity of *B. cinerea* could not be attributed to enzyme action. Two metabolites, botrydial and dihydrobotrydial, isolated from the culture filtrate appeared to be responsible for the phytotoxic effect.

INTRODUCTION

Botrytis cinerea is a grey mould which causes disease on many flowers, fruits and vegetables. Economically important crops such as lettuces, carrots, tobacco and grapes are attacked by this fungus [1]. Considerable effort has been devoted to elucidating the mechanism by which *Botrytis* infects plants. Published results implicate fungal enzymes such as cutinases, proteinases and/or polygalacturonases [2–4] in this process and plant glucanases, chitinases and phytoalexins [5, 6] in plant resistance to the fungus.

The widespread use of chemical fungicides in the prevention or elimination of fungal attack has resulted in serious problems as a result of the appearance of highly resistant strains, contamination of soil and water owing to fungicide persistence and serious economic damage because of the decreased quality of wines produced from treated grapes [7]. Genetically modified plants that are resistant to fungal attack and thus dispense with the need to use fungicides have been reported. Transgenic tobacco plants have been reported which possess an enhanced protection against fungal attack through the overexpression of phytoalexins [8] or the constitutive coexpression of chitinase and glucanase genes [9].

However, in the case of some plants such as the grapevine, the replacement of local crops by transformed ones is not feasible in the short term. These plants are perennial and there are local varieties that are very well adapted to local ecoclimatic conditions and produce specific wines that are commercially protected.

An alternative way of overcoming the problem would be the use of selective fungicides that act on specific targets. This requires a knowledge of the fungal metabolites responsible for the damage to the plants and their biosynthesis [10, 11]. Therefore, we have begun a research programme directed at understanding the role of botrydial (**1**) and structurally-related compounds in the expression of the phytotoxicity of *Botrytis cinerea* and its subsequent development. This is, to our knowledge, the first report of the *in vitro* production of the symptoms of the grey mould disease and it could open the way to the use of selective fungicides against *Botrytis*.

RESULTS AND DISCUSSION

The fungus *B. cinerea* is a known pathogen of tobacco [12] and our strain (UCA 992), although isolated from grapes, was also pathogenic to tobacco, which provided a reliable test system.

Phytotoxic activity produced by *B. cinerea* was detected by incubating aliquots of fungus-free culture filtrate on 1-cm diameter tobacco leaf discs. The results are summarized in Table 1. Phytotoxic activity was detected in fermentations grown for 2 days, for 10–15 days and for 22 days (final assay). The filtrates from day 2 to day 10 had no effect. The leaf circles were examined each day; the phytotoxic effect reached a maximum on the seventh day and remained constant thereafter. The results for the seventh day are shown in Table 1.

The phytotoxic effect caused by the fungus-free filtrate was the same as that produced on the leaves by the spores and the mycelium. The phytotoxic metabolites were therefore extracellular.

The interaction between the host plant and the

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Table 1. Bioassay of culture static filtrates from static fermentation

Day of fermentation	% Affected circles		% Affected surface*	
	Exp. 1†	Exp. 2	Exp. 1	Exp. 2
2	80	73	7.4	8.7
5‡	0	0	0	0
8	7	0	0.5	0
10	26	20	1.9	14.0
11	73	70	7.4	18.3
12	80	67	7.9	14.4
13	93	93	19.1	30.3
14	100	80	14.0	15.1
15	100	100	24.9	72.6
22	100	100	49.0	81.4

*Calculated as the surface affected compared to the total surface expressed in mm^2.

†Results from two independent experiments with 15 circles of tobacco leaves for each day in each experiment.

‡Days 5 and 8 are both negative.

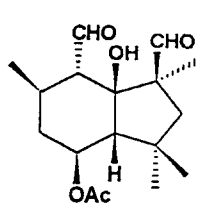
pathogen is not clear. The killing of the plant cells by necrotrophic fungi has been attributed to various compounds, hormones [13], enzymes [2–4, 14, 15] and to changes in ionic permeability [16]. Published results reveal several stages in the enzymatic effects involved in the *Botrytis* infection [2–4]. Initially, fungal enzymes macerate the host tissue allowing establishment and invasion by the fungus. This hypothesis is supported by the general observation that the natural wounding or maceration of tissue produced by insect bites, in rain or hail, dramatically increases invasion by *Botrytis*. The second step involves the production of low M_r metabolites that are responsible for the visible symptoms of the plant disease.

The filtrates from days 2, 12, 13, 15 and 22 were boiled for 20 min and then bioassayed. The results were the same as those from the crude extracts indicating that the phytotoxic effect was caused by low M_r metabolites. Kaile *et al.* [16] observed that high levels of calcium and other ions released from the plant cells was as a result of the maceration of the host tissue by the pectolytic enzymes secreted by fungi. These ions could be involved in host cytoxicity during necrotrophic pathogenesis. According to our data, the phytotoxic effect is produced *in vitro* in the absence of enzymes. However, *in vivo* the presence of at least some enzymes may be necessary for fungal invasion. Although calcium ions may be involved, they do not appear to be the major agents responsible for the disease symptoms.

Further work was carried out to isolate the phytotoxic metabolites and to quantify their effect. Extracts from 14-, 15- and 22-day-old fermentations, which showed high activity, were separated by chromatography. The details of which have been published [17]. The neutral fraction was split into six subfractions. Those fractions showing biological activity were analysed by TLC and purified by normal-phase HPLC to yield botrydial (**1**), dihydrobotrydial (**2**) and botrydienal (**3**).

The chromatographically pure metabolites were bioassayed and it was found that botrydial (**1**) and dihydrobotrydial (**2**) produced the phytotoxic effect of the fungus-free filtrate. The effect at several concentrations is shown in Table 2. Botrydienal (**3**) was present at very low concentration in the culture and insufficient material for a bioassay was purified.

The kinetics of the production of the metabolites by *B. cinerea* were studied. The variation of pH, mycelial dry weight, botrydial (**1**) and dihydrobotrydial (**2**) concentration with time is given in Table 3. There is a correlation between the occurrence of the grey mould symptoms and the time of production of botrydial (**1**) and dihydrobotrydial (**2**). Although there was some phytotoxic activity produced by the fungus on day 2, we were unable to purify any metabolite that reproduced the symptoms at this stage of the fermentation. It was not until day 10 of the fermentation when the dihydrobotrydial (**2**) concentration was 6 $\mu g\,ml^{-1}$ and the botrydial (**1**) concentration was 1 $\mu g\,ml^{-1}$ that the phytotoxic activity appeared. The activity then increased in step with the concentration of these



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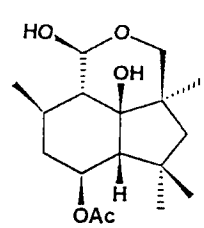

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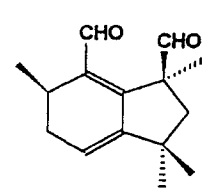

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Table 2. Qualitative and quantitative effect of isolate metabolites on day 7 of bioassay

Metabolite concentration (ppm)	% Affected circles		% Affected surface*	
	Exp. 1†	Exp. 2	Exp. 1	Exp. 2
Botrydial				
125	100	98	27.6	30.0
60	95	94	10.7	9.5
30	83	80	6.1	5.0
10	41	43	3.2	3.8
1†	32	30	1.4	2.5
Dihydrobotrydial				
1000	25	48	11.2	7.6
500	20	20	4.0	5.4
250	20	0	2.5	0
200†	20	0	0.9	0

*Results from two independent experiments with 25 circles for each concentration in each experiment.

‡Minimal effective concentration.

metabolites, reaching a maximum on day 22, when the concentration of dihydrobotrydial (**2**) had tripled and botrydial (**1**) had doubled. However, the mycelial dry weight remained constant from day 13, indicating that the culture reached the stationary phase. The metabolites are thus typical secondary metabolites of the fungus, supporting the idea that there are two phases in the production of grey mould symptoms. The relatively high amounts (125 and 1000 ppm) of botrydial (**1**) and dihydrobotrydial (**2**) necessary to separately reproduce an equivalent level of symptoms compared with the concentrations (13 ppm for botrydial, 46 ppm dihydrobotrydial) in the fungus-free culture filtrates suggests that they, or a hitherto undetected third metabolite, may have a synergistic effect.

The effect of the glucose concentration on the production of botrydial (**1**) and dihydrobotrydial (**2**) was examined (Table 4). There was still some glucose present (14.13 $g\,l^{-1}$) when their production was at a maximum. In order to investigate the effect of glucose concentration and to see if there is any catabolic regulation of the biosynthesis of these metabolites by glucose, as found in the case of some antibiotics [18, 19], we incubated *Botrytis* at four different glucose concentrations. The results (Table 4) show that glucose does not catabolically regulate phytotoxic metabolite production and, indeed, some residual glucose in the medium is needed for their production.

In conclusion, it can be seen from this data that a visible phytotoxic effect of *B. cinerea* can be reproduced *in vitro* by **1** and **2**. Without excluding other factors, this provides circumstantial evidence for a

Table 3. Kinetic parameters and quantification of DHB* and botrydial from static culture of *Botrytis cinerea*

Day of fermentation	pH	Biomass† ($mg\,ml^{-1}$)	DHB ($\mu g\,ml^{-1}$)	Botrydial ($\mu g\,ml^{-1}$)	Residual glucose ($g\,l^{-1}$)
2	6.15	0	0	0	50.00
3	5.80	0.31	0.50	0.25	34.91
4	4.77	2.33	0.75	0.25	34.70
5	4.62	3.02	0.75	0.50	34.14
6	4.48	3.45	2.50	0.50	34.25
7	4.56	3.71	3.50	–‡	34.55
8	4.61	3.65	4.00	–	34.29
9	4.59	3.56	4.50	1.00	34.19
10	4.51	3.84	6.00	1.00	33.57
11	4.33	4.10	7.50	1.00	14.68
12	4.37	4.37	8.00	–	14.68
13	4.00	6.82	8.50	3.00	14.67
14	3.92	6.72	10.50	4.50	14.65
15	3.79	6.61	16.50	6.50	13.58
22	3.56	6.61	46.50	13.00	13.13

*DHB: dihydrobotrydial.

†Biomass calculated as mycelium dry weight by volume of filtrate.

‡Not determined.

Table 4. Bioassay of several glucose concentrations upon growth and infection by *Botrytis*

Glucose concentration ($g\,l^{-1}$)	Day*	% Circles affected	% Surface affected
10	10	0	0
	20	0	0
20	10	0	0
	20	0	0
30	10	90	20.3
	20	85	21.6
40	10	75	12.8
	20	85	36.1
50	10	95	18.6
	20	90	61.8

*Results from bioassays show effects at 8th day. Bioassay was performed with 40 circles for each glucose concentration, 20 were used for the culture filtrate from day 10 and 20 for day 20.

putative role for these compounds in the pathogenicity of the organism *in vivo*. Qualitatively, we have observed that other strains which produce less botrydial and dihydrobotrydial, are also less bioactive.

EXPERIMENTAL

The strain of *B. cinerea* UCA 992 employed in this work is deposited in the Mycological Herbarium Collection of the Facultad de Ciencias at the Universidad de Cádiz (UCA). It had been isolated from grapes at a Domecq vineyard (Jerez, Cádiz, Spain).

Screening for phytotoxic activity. The fungus was grown in Roux bottles containing 200 ml of a modified Czapeck–Dox medium (5% glucose, 0.1% yeast extract, 0.05% KH_2PO_4, 0.2% $NaNO_3$, 0.05% $MgSO_4 \cdot H_2O$ and 0.001% $FeSO_4 \cdot 7H_2O$). Each bottle was inoculated with 100 μl of conidia containing 2.7×10^7 conidia ml^{-1}.

Aliquots were taken from the Roux bottles after 1–15 and 22 days of growth of *B. cinerea* and kept frozen at −20° until used. The samples were centrifuged for 30 min at 20 000 *g* before use in order to eliminate the condidia and the remainder of the mycelium.

Bioassays. Tobacco leaves from *Nicotiana tabacum* var. Xanthi NC were used. The leaves were sterilized with 10% $NaClO_4$ for 3 min, washed (×4) with sterile H_2O, dried between filter papers, cut into circles 1-cm diam. and placed in Petri dishes containing Whatman paper wetted with sterile H_2O. Extract, 10 μl, was placed on each of the circles and the plates were kept at 25–28°.

The purified metabolites were dissolved in 40% aq. Me_2CO containing aq. Tween 80 (10 μl Tween 80 in 100 ml water). Controls consisted of sterile, uninoculated growth medium and the mixtures of Me_2CO–H_2O–Tween 80 used for dissolving the purified metabolites or frs. Qualitative results were obtained by counting the number of circles affected compared with the total. Quantitative results were obtained by determining the surface area affected compared with the total surface area.

Kinetic studies. The fungus was grown in 19 Roux bottles. The cultures were filtered on days 1–15 and 22. Aliquots were taken, the pH measured and the residual glucose determined [20]. The biomass was measured from the wt of dry mycelium. The mycelium that was collected by filtration was placed between sheets of Whatman paper in an oven at 100° overnight and then weighed.

Isolation of fractions and metabolites. The fungus was grown on Czapeck–Dox medium (5.2 l) 26 Roux bottles. The procedures used for the fractionation and isolation of the metabolites are described in ref. [17].

The effect of glucose concentration. Czapeck–Dox media containing 10, 20, 30, 40 and 50 $g\,l^{-1}$ glucose were prepared. Two Roux bottles were incubated at each concentration, one was harvested after 10 days and the other after 20 days growth.

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