The biodegradation of the phytotoxic metabolite botrydial by its parent organism, *Botrytis cinerea*

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The inhibition of the growth of *Botrytis cinerea* has been found to be directly proportional to the concentration of its metabolite, botrydial (**1**). The fungus transforms botrydial (**1**) to the less active phytotoxins dihydrobotrydial (**2**), botryenedial (**3**), and secobotrytrienediol (**4**). Two main biodegradative pathways of botrydial (**1**) and the interconversion of the botryane toxins excreted by *B. cinerea* are proposed.

Keywords: botryane, phytotoxins, metabolism, *Botrytis cinerea*, growth inhibition

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.[1] A number of phytotoxins have been isolated from this fungus.[2] The best known and most active of these metabolites is botrydial (**1**) which possesses the sesquiterpenoid botryane skeleton.[3] It is responsible for the typical lesions of the fungal infection and it plays an important role in the pathogenicity of the organism *in vivo*.[4,5] Botrydial (**1**) has recently been detected in ripe fruits of *Capsicum annuum* and in the leaves of *Phaseolus vulgaris* and *Arabidopsis thaliana* which have been wounded and inoculated with a conidial suspension of *B. cinerea*.[6]

The fungus also produces different amounts of less active metabolites (**3**–**7**) during the fermentation.[2] The structures of these metabolites suggest that a number may arise from the biotransformation of botrydial (**1**). Recently reported experiments[7] with labelled substrates, indicated that secobotrytrienediol (**4**) is biosynthesised from farnesyl diphosphate by the same folding as has been described[8] for the biosynthesis of botryanes. This led to the suggestion that this compound could be arising from a branch in botryane biosynthesis. In this paper we describe the regulatory effect of botrydial (**1**) on the growth of *B. cinerea* and its possible biodegradation by the fungus.

Botrydial (**1**) is a particularly powerful phytotoxin. It has been shown to have phytotoxic and cytotoxic activity at concentrations as low as 1 ppm[4,5,9] and antibiotic activity at 100 ppm.[10] In order to examine the inhibition of the growth of *B. cinerea*, the fungus was incubated in both solid and liquid culture with exogenous botrydial (**1**). In the absence of botrydial (**1**) on a solid medium, the mycelium had completely covered the surface of a Petri dish by the sixth day. However, in the presence of botrydial (**1**) the growth was inhibited in proportion to its concentration. Growth ceased at 250 ppm (Fig. 1). This metabolite, therefore, has a regulatory role on the growth of its parent organism.

Botrydial (**1**) was added to the fungus after two days growth on liquid medium when the fungus was in the exponential growth phase. The rate of increase of biomass decreased as the amount of botrydial (**1**) that was added to the medium increased (Fig. 2). However, 24 hours after the addition of botrydial (**1**), the fungal growth slowly resumed. This suggested that botrydial (**1**) was being converted to less inhibitory metabolites.

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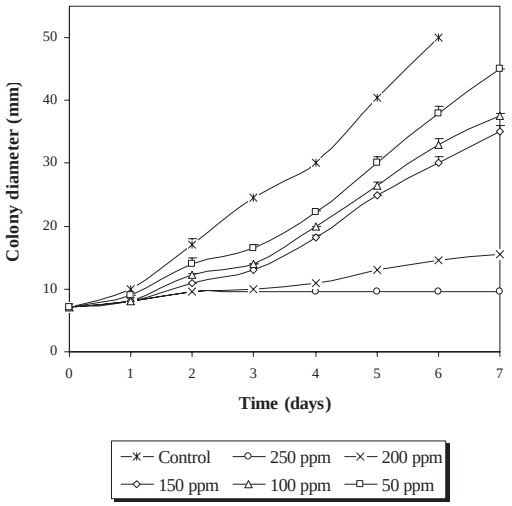


Fig. 1 Effect of botrydial (**1**) on the growth of *B. cinerea* on solid medium. Represented data are the arithmetic mean and standard deviations of the results from two independent experiments.

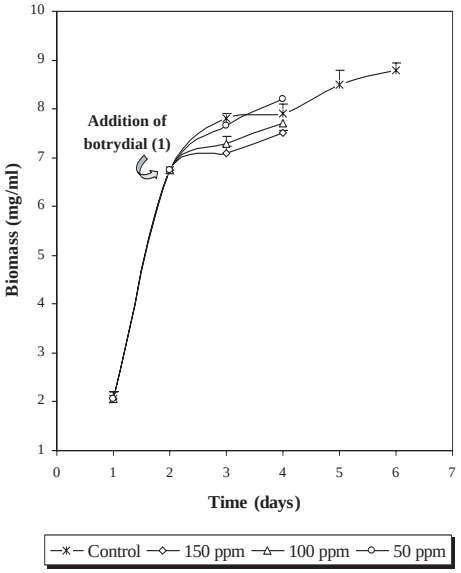


Fig. 2 Effect of botrydial (**1**) on the growth of *B. cinerea* on liquid medium. Represented data are the arithmetic mean and standard deviations of the results from two independent experiments.

We then examined the formation and metabolism of the less harmful metabolites dihydrobotrydial (**2**),[3] botryenedial (**3**)[4] and secobotrytrienediol (**4**)[12] during the various stages of growth of *B. cinerea*. The fungus was grown in 16×500 ml flasks each containing 200 ml Czapek-Dox medium. The mycelium from two flasks was separately filtered on each of days 1–8 and the broth extracted with ethyl acetate. The four metabolites (**1–4**) were separated by HPLC and identified. The fungal biomass was monitored by measuring the weight of the freeze-dried mycelium. The pH and glucose levels in the medium were also measured. The results are shown in Fig. 3.

Botrydial (**1**) production started on the second day of the fermentation when the fungus was still in the exponential growth phase and it reached a maximum on the fifth day. Although this metabolite begins to be formed during the exponential growth phase, it is a typical secondary metabolite in that it is obtained principally during the stationary phase. In contrast, the production of dihydrobotrydial (**2**) was correlated with the increase in biomass and by the day 8 there was a high concentration arising from a progressive accumulation during the fermentation. Earlier biosynthetic results had shown[8] that botrydial (**1**) is converted into dihydrobotrydial (**2**).

Although botryenedial (**3**) is less phytotoxic than botrydial (**1**), it still produces the characteristic necrotic lesions of the plant disease.[11] The production of botryenedial (**3**) began on the second day of the fermentation and it reached a maximum on the fifth day, coinciding with the maximum amount of botrydial (**1**). It may be formed from the latter and indeed botrydial (**1**) can be transformed into **3** by treatment with oxalic acid.[11]

The production of the moderately phytotoxic metabolite secobotrytrienediol (**4**)[12] occurred between days 2 and 5. Whilst the concentration of botrydial (**1**) increased rapidly until it reached a maximum on day 5, the concentration of **4** reached a maximum on day 4 and it had almost disappeared by day 5. A detailed study of Fig. 3 suggested that the production of **4** could be regulated by the pH of the medium. Some interesting results were obtained by varying the pH of the fermentation. Whilst botrydial (**1**), dihydrobotrydial (**2**) and its derivatives were formed when the pH of the broth was acidic, only secobotrytrienediol (**4**) was found when the pH was close to 7.

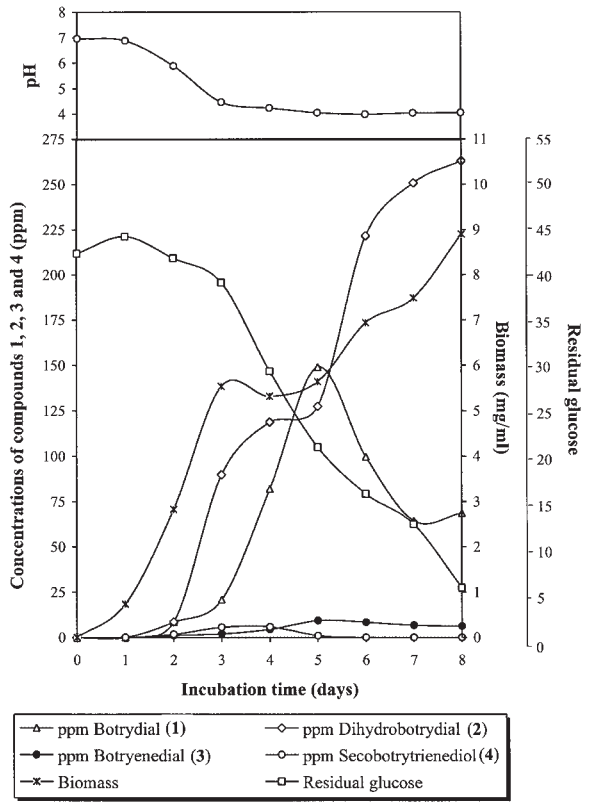


Fig. 3 Biomass, residual glucose, pH and production of phytotoxins **1**-**4** of a *B. cinerea* liquid culture. Represented data are the arithmetic mean of the results from two independent experiments.

These results involving the co-occurrence of compounds **1–4**, suggest that they may be inter-related as shown in Scheme 1. In this scheme which interlinks the major botryane metabolites, the sequential elimination of the hydroxyl and acetoxyl groups of botrydial (**1**) is favoured since it extends conjugation of the C-10 aldehyde. The C-15 aldehyde becomes part of a vinylogous β-diketone leading to the loss of one formyl group (**5**). Retroaldol reactions involving the C-9 alcohol may lead to epimers at this centre. The formation of secobotrytrienediol (**4**) may involve an electrocyclic ring opening reaction from diene **9**.

In conclusion we have shown that the phytotoxin botrydial (**1**) which is produced during the initial stages of the growth of *B. cinerea*, plays an important role in the life cycle of the fungus and regulates its growth at high concentrations. After its metabolism to the less inhibitory compounds, growth resumes. This may have implications for our understanding of the progress of the fungal infection of a plant. There appear to be at least two main biodegradative and detoxification pathways for botrydial (**1**) (routes a and b in Scheme 1).

Experimental

General experimental procedures. These were identical to those previously described.[12]

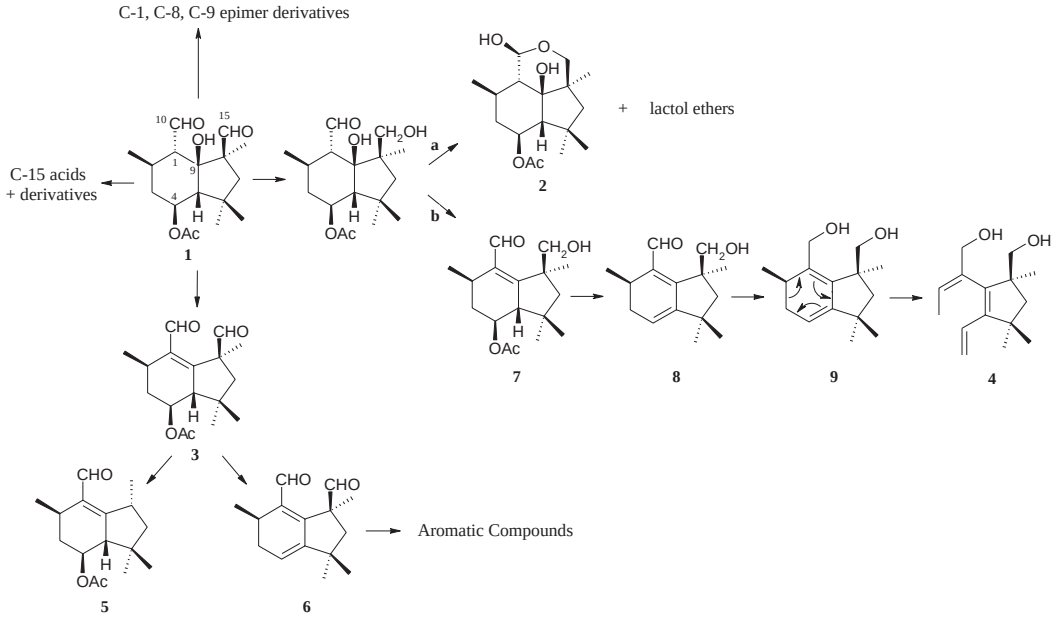
Fungal strain: The strain 2100 of *B. cinerea* used in this research was obtained from the Colección Española de Cultivos Tipo (CECT), Facultad de Biología, Universidad de Valencia, Spain. Conidial stock suspensions of this strain were maintained viable in 80% glycerol at -20 °C.

Inhibition assays with botrydial (1): Assays on the solid medium were performed following the "poisoned food technique".[13] The test compound was dissolved in EtOH to give final concentrations ranging from 50 to 250 ppm. Solutions of the test compound were then added to a glucose-malt-peptone-agar medium (61 g of glucose-malt-peptone-agar per litre, pH 7.0). The final EtOH concentration was identical in both the control and treatment groups. The medium was poured into Petri dishes measuring 6 cm in diameter and a mycelial disk (7 mm diameter) of B. *cinerea* cut from an actively growing culture was placed in the centre of the agar plate. Inhibition of radial growth was measured for 7 d.

Assays in the liquid medium were conducted as below and monitored by weighing the freeze-dried mycelium. After 48 h of incubation the culture was poured into four flasks (250 ml) each containing 50 ml of Czapek-Dox medium and a solution in EtOH of the test compound at final concentrations ranging from 50 to 150 ppm. One flask was used as a control. The final EtOH concentration was identical in both control and treatment groups. The freeze-dried mycelium was weighed each day for 6 d in the control and for 4 d in the assay with botrydial (1).

Culture conditions and parameters of fungal growth: The fungus was grown in 16 flasks (500 ml) containing 200 ml of Czapek-Dox medium.[12] The pH of the medium was adjusted to 7.0. Each flask was inoculated with 1.1×10^7 fresh conidia and then incubated at 24–26°C on an orbital shaker at 250 rpm. The mycelium was separated from the culture broth by centrifuging the cultures for 5–10 min on days 1–8. Aliquots, taken from the broth in order to determine the residual glucose, were kept frozen at -20 °C until they were used. The pH was measured and botrydial (**1**) and its derivatives **2**, **3**, and **4** were purified and quantified. The biomass was determined from the weight of the freeze-dried mycelium. The measurements were taken twice.

Residual glucose determination: To ascertain the amount of residual glucose, the phenol-sulphuric acid assay was used.[14] Thus, 0.5 ml of an aqueous solution of the broth in the range of concentrations of 10–300 ppm was mixed with 1 ml of an aqueous solution of 5% phenol. H_2SO_4 (2.5 ml) was then added to the



Scheme 1 Proposed detoxification mechanisms of botrydial (**1**).

solution, which led to an increase in temperature. After 5 min the solution was cooled and the absorbance measured twice at 490 nm.

Purification and quantification of toxins 1–4: The broth was acidified to pH 2 with HCl, saturated with NaCl, and extracted (x3) with EtOAc. The organic extract was washed (x3) with a saturated aqueous solution of $NaHCO_3$ and H_2O, dried over anhydrous Na_2SO_4, and evaporated under reduced pressure. The crude extract was separated by means of column chromatography over silica gel with hexane-EtOAc (7:3) as the solvent. Semipreparative HPLC was used to further purify the collected fraction to yield botrydial (**1**), dihydrobotrydial (**2**), botryenedial (**3**), and secobotrytrienediol (**4**).

Fermentation on buffered medium: The fungus was grown in four flasks containing Czapek-Dox medium under the conditions described above. The mycelia were transferred, after 3 d, into 2 flasks containing 200 ml of Czapek-Dox medium buffered at pH 7 with phosphate buffer 0.4 M. An additional two flasks were used as control. The broth and the mycelia were separated by filtration after a further 3 d. The pH was measured (control: 4.4 pH units; buffered broth: 6.3 pH units) and the broth was saturated with NaCl, and extracted as above. Both extracts were studied by thin-layer chromatography showing the presence of **1**, **2** and **3** in the control, and only **4** in the buffered broth.

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