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# Botrydial is produced in plant tissues infected by *Botrytis cinerea*

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**Abstract**

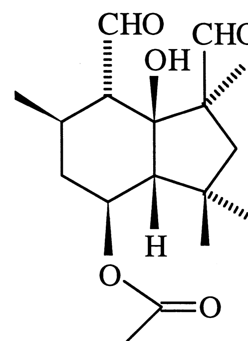
The fungal metabolite botrydial was detected for the first time in ripe fruits of sweet pepper (*Capsicum annuum*) wound-inoculated with conidial suspensions of *Botrytis cinerea* and also in leaves of *Phaseolus vulgaris* and *Arabidopsis thaliana* inoculated without wounding. This phytotoxin was produced in soft rot regions of the infection. In *C. annuum*, the most aggressive isolate produced the highest botrydial concentrations *in planta*. The levels of botrydial produced by this isolate did not correlate with the reported relative susceptibilities of four *P. vulgaris* genotypes. The results suggest that botrydial is a pathogenicity factor for this fungus, but not a primary determinant of pathogenicity. © 2001 Elsevier Science Ltd. All rights reserved.

*Keywords:* *Botrytis cinerea*; Fungal metabolites; Phytotoxicity; *Capsicum annuum*; *Phaseolus vulgaris*; *Arabidopsis thaliana*

## 1. Introduction

A number of factors have been suggested as the primary causes of lesion development in grey mould disease caused by *Botrytis cinerea*, a ubiquitous necrotrophic pathogen affecting more than 200 crops, especially the leaves, flowers and fruits of plants of horticultural importance (Coley-Smith et al., 1980). The pathogen secretes numerous enzymes and other low molecular weight compounds that have been implicated in pathogenicity. These include cutinases (van Kan et al., 1997), cell wall degrading enzymes (Leone and van den Heuvel, 1987; Johnston and Williamson, 1992; Elad and Evensen, 1995; ten Have et al., 1998), aspartic proteases (Movahedi and Heale, 1990), oxidative enzymes and oxalic acid involved in production of active oxygen species (Edlich et al., 1989; von Tiedemann, 1997; Deighton et al., 1999; Muckenschnabel et al., 2001).

The pathogen also produces a number of toxic bicyclic sesquiterpenes with the botrydial skeleton in static liquid culture (Collado et al., 1995, 1996; Rebordinos et al., 1996; Durán-Patrón et al., 1999, 2000). In bioassays with floating tobacco leaf disks and attached leaves, purified botrydial (**1**) applied to the adaxial leaf surface was phytotoxic (Rebordinos et al., 1996). However,




**(1)**

botrydial and these other metabolites have never been identified in plant tissues infected with isolates of *B. cinerea*. A greater understanding of the processes leading to disease onset and lesion expansion would be gained if this metabolite could be detected in infected whole plant tissues, at concentrations known to be phytotoxic in leaf disk assays (Rebordinos et al., 1996).

This paper describes experiments designed to determine whether botrydial has a role in pathogenicity of the grey mould pathogen. Sweet pepper (*Capsicum annuum*) fruits can be severely affected by the disease in glasshouse production and *B. cinerea* consistently produces

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large spreading soft rot lesions in the pericarp of these fruits that are amenable to chemical analysis when inoculated with the pathogen (Deighton et al., 1999). Therefore peppers were inoculated with three different fungal isolates and analysed for botrydial. Analyses were also made from leaves of French bean (*Phaseolus vulgaris*) and *Arabidopsis thaliana* inoculated with a single aggressive isolate.

## 2. Results and discussion

All three isolates produced substantial soft rot lesions in *C. annuum* after wound inoculation, the largest lesions forming within 6 days in attached fruits inoculated with isolate B05.10. Botrydial (**1**) was detected in peppers, but its appearance and concentration was isolate-dependent and varied considerably between individual fruits (Table 1). A typical chromatogram from a soft rot lesion of a pepper inoculated with isolate B05.10 is shown in Fig. 1A, and that from a non-inoculated pepper in Fig. 1B. The internal standard (DNP-FBz) elutes at ca. 22 min (*m*/*z* 303), the 2,4- dinitrophenylhydrazone of botrydial ($DNP_2$-bot) elutes at ca. 26 min (*m*/*z* 669).

In attached leaves of *P. vulgaris* inoculated with the most aggressive *B. cinerea* isolate, B05.10, all leaves in the experiment became infected and botrydial was detected in all lesions (Table 1). In *A. thaliana*, the compound was detected in only three of the eight infected leaves examined and at lower levels than in the other two host tissues.

This is the first evidence that botrydial can be produced *in planta*. The fact that the same aggressive isolate released the compound in attached tissues of members of the Solanaceae, Leguminoseae and Brassicaeae grown under controlled conditions suggests that botrydial has some role in pathogenicity. However, although large soft rot lesions were always produced, the compound was detectable only in 50% of peppers inoculated with one isolate (B1.19), and was never detected in these experiments with another (B1.4). It is possible that botrydial was present in these samples, but at concentrations lower than the current detection limit (0.04 nmol $g^{-1}$).

Table 1
Occurrence of botrydial in tissues of *Capsicum annuum*, *Phaseolus vulgaris* and *Arabidopsis thaliana* infected by *Botrytis cinerea*

| Host[a] | Genotype | Isolate | No. positive samples | Botrydial conc. (range nmol $g^{-1}$) |
|---|---|---|---|---|
| *Capsicum annuum* | Bendigo | B05.10 | 7/7 | 0.44–12.0<br>**2.8**[b] |
| | Bendigo | B1.19 | 4/8[c] | 0.080–0.34<br>**0.17**[b] |
| | Bendigo | B1.4 | 0/8[c] | nd |
| *Phaseolus vulgaris* | Huron | B05.10 | 18/18 | 7.6–260<br>**140**[b] |
| | 164214 | B05.10 | 9/9 | 0.23–18<br>**7.7**[b] |
| | N90598 | B05.10 | 18/18 | 5.8–120<br>**38**[b] |
| | 416664 | B05.10 | 14/14 | 4.0–35<br>**11**[b] |
| *Arabidopsis thaliana* | CS2360 | B05.10 | 3/8[c] | 0.097–0.48<br>**0.28**[b] |

[a] Four days post inoculation (*C. annuum, P. vulgaris*), 6 days (*A. thaliana*).
[b] Mean value in tissues where detected; nd, not detected.
[c] Limit of detection = 0.04 nmol $g^{-1}$ (fresh weight), therefore botrydial may be present at <0.04 nmol $g^{-1}$.

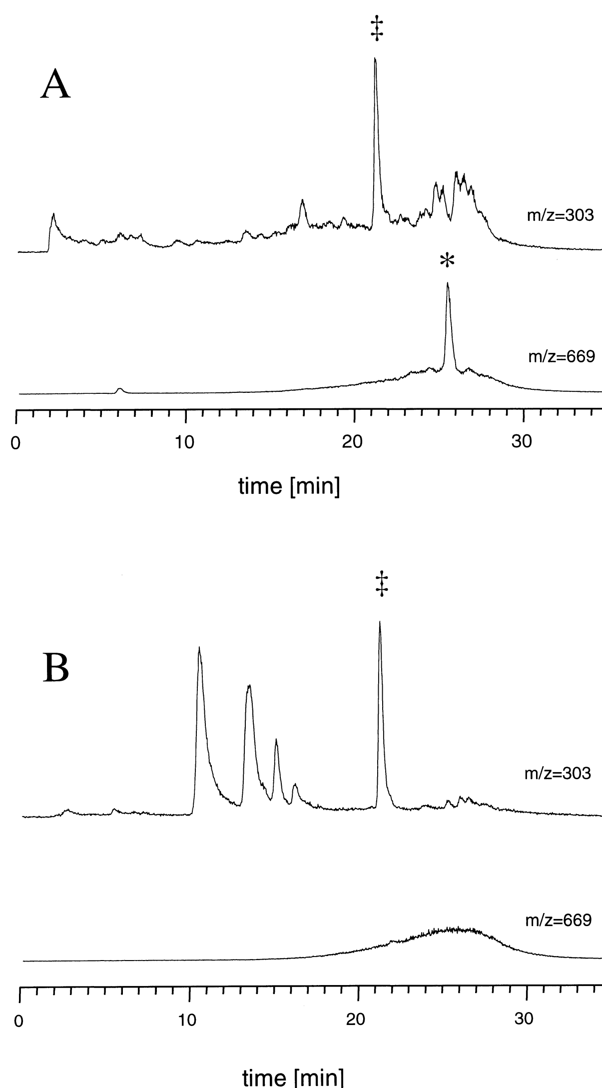


Fig. 1. Typical chromatograms from A: a soft rot lesion in the pericarp of a *Capsicum annuum* fruit inoculated with *Botrytis cinerea* isolate B05.10, and B: a non-inoculated control. Upper panels: *m*/*z* 303 ion trace. Lower panels: *m*/*z* 669 ion trace. Internal standard (DNP-FBz, ‡) elutes at ~22 min (*m*/*z* 303), botrydial derivative ($DNP_2$-bot, *) elutes at ~26 min (*m*/*z* 669).

These results suggest that isolates differ in their ability to produce substantial quantities of botrydial *in planta*.

Inoculation of four genotypes of *P. vulgaris* with the most aggressive *B. cinerea* isolate (B05.10) resulted in the accumulation of appreciably higher mean concentrations of botrydial than in *C. annuum*, the highest levels occurring in cv. Huron (140 nmol $g^{-1}$) and with very large differences between samples. There was no apparent relationship between botrydial concentration and lesion size, or the reported relative resistance of the genotypes to ozone toxicity — which in turn correlates with resistance to *B. cinerea* (von Tiedemann, 1997). In the case of the genotype Huron, botrydial was detected at concentrations 50-fold higher than those reported (Rebordinos et al., 1996) to be phytotoxic when tested in vitro on tobacco leaf disks (i.e. 1 ppm). Attempts to detect the compound at earlier stages in the infection may be difficult because of the sample size required for LC–MS. Another approach would be to take a series of samples across a leaf with a spreading soft rot lesions to indicate when production of the toxin begins in relation to appearance of lesions. Other compounds with the botryane skeleton may also be present in tissues, but the methodology applied in the present study is only applicable to aldehydic derivatives. Results therefore suggest that botrydial is associated with pathogenesis, but is not a primary determinant of the disease process.

## 3. Experimental

### 3.1. Plant material and methods of culture

Plants were grown from seeds in a standardised and quality controlled compost/sand mixture and given a minimum photoperiod of 14 h light at 20°C, with supplementary lighting provided by Hg-discharge lamps as described elsewhere (Muckenschnabel et al., 2001). Plants of *Capsicum annuum* cv. Bendigo were grown in the glasshouse to produce green fruits in excess of 10 cm in length and received no fungicide or insecticides. Seeds of *Phaseolus vulgaris* genotypes Huron (Kelly et al., 1994) and N90598 (from J.D. Kelly, Michigan State University, East Lansing, MI, USA) and 164214 and 416664 (from P.S. Benapal, Virginia State University, Petersburg, VA, USA) were grown under similar conditions to produce fully expanded primary leaves for inoculation. Seeds of *Arabidopsis thaliana* (CS2360 Wassilewskija, Ohio State University, Columbus, USA) were also grown under these conditions.

### 3.2. Fungal inoculum and inoculations.

Three isolates of the fungus *Botrytis cinerea* Pers.: Fr. [teleomorph *Botryotinia fuckeliana* (de Bary) Whetz.] were used in inoculations. Isolate B05.10 (Quidde et al., 1999), a haploid aggressive strain, was used on all plant hosts. Isolates B1.19 and B1.4 from *Vitis vinifera* were used only to inoculate pepper fruits. Conidial stock suspensions of all isolates remained viable in 75% glycerol at −80°C. Inoculum was obtained from 14-day-old cultures on potato dextrose agar containing an homogenate of leaves from *Phaseolus vulgaris* (50 g $l^{-1}$ of media) and conidial suspensions at $1\times10^5$ conidia $ml^{-1}$ were prepared as previously reported (Muckenschnabel et al., 2001).

In the glasshouse, attached pepper fruits were inoculated with 5 µl of the conidial suspension placed inside a tangential wound made by sterile scalpel in the proximal region. The adaxial surface of fully expanded leaves of *P. vulgaris* and *A. thaliana* (14 days old) were inoculated with two or one interveinal droplets (5 µl) of conidial suspension, respectively, and incubated in closed propagation boxes at high humidity for 4 days (*C. annuum*, and *P. vulgaris*) or 6 days (*A. thaliana*) as described by Muckenschnabel et al. (2001).

### 3.3. Sampling, extraction and analysis

The soft rotted areas of the fruit and leaves were excised, weighed and extracts prepared for LC–MS analyses. Briefly, leaf tissue (approx. 100 mg) was homogenised with methanol containing BHT (0.2 mM) as antioxidant (1 ml) in a MiniBead Beater (Biospec Products, Bartlesville, OK, USA) using a mixture of 0.5 g of 0.1 mm diameter and 0.5 g of 0.5 mm diameter glass beads. The methanolic extracts were then derivatised with 2,4-dinitrophenylhydrazine (35 mg 100 $ml^{-1}$ in 1 M HCl) over a period of ca. 30 min at ca. 20°C. The dinitrophenylhydrazones (DNPHs) were extracted from the reaction mixture into dichloromethane (2×2 ml) and dried under nitrogen (40°C) in a Reacti-Therm heating module (Pierce, Rockford, IL, USA). These extracts were then made up in acetonitrile (200 µl) containing 1 µM of the DNPH-derivative of 4-fluorobenzaldehyde (DNP-FBz) as internal standard.

LC–MS analyses were carried out on a Finnigan MAT SSQ710C single quadrupole mass spectrometer with an APCI interface (ThermoQuest, Hemel Hempstead, UK) in negative ion mode with nitrogen as the reagent gas. The samples (5 µl) were eluted from a Hypersil C18 column (250×2 mm, Phenomenex, Macclesfield, UK) with a gradient from 60–90% acetonitrile in 40 mM ammonium acetate. Detection was by selected ion monitoring of the 2,4-dinitrophenylhydrazone derivatives of botrydial ($DNP_2$-bot, $[M-H]^-$ $m/z$ 669) and 4-fluorobenzaldehyde (DNP-FBz, $[M-H]^-$ $m/z$ 303). Alternate blank injections were made to ensure no carry-over from sample-to-sample. Quantification was made from a calibration curve (0.04–1250 nmol $g^{-1}$ leaf tissue, fresh weight) prepared by injection of authentic standards (Collado et al., 1995, 1996) derivatised as above.

A second chromatographic system of different selectivity was used to ensure that the analyte from *B. cinerea*-inoculated hosts co-eluted with the authentic standard. A LUNA hexyl-phenyl (Phenomenex) column (150×2mm) was eluted with a linear gradient of 50–80% acetonitrile containing 40 mM ammonium acetate (25 min gradient, then 80% acetonitrile isocratic). With this chromatographic system, extracts from inoculated host plants gave rise to a peak (*m*/*z* 669) at approx. 28 min that co-eluted with that of DNPH-derivatised botrydial (results not shown).

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