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THE PUTATIVE ROLE OF BOTRYDIAL AND RELATED METABOLITES IN THE INFECTION MECHANISM OF *Botrytis cinerea*

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Abstract—Phytotoxic assays, performed both *in vitro* and *in vivo* on leaves of *Phaseolus vulgaris*, with metabolites excreted by the fungus *B. cinerea* are evaluated. Exogenous application of the phytotoxin botrydial has been found to produce severe chlorosis and cell collapse and facilitated fungal penetration and colonization of plant tissue. The results also show a light-dependent action mechanism for the phytotoxin and seem to indicate that botrydial is a non-host-specific toxin involved in fungal infection of *B. cinerea*.

Key Words—Botryane, botrydial, chlorosis, phytotoxin, *Botrytis*, virulence factor.

INTRODUCTION

A substantial number of toxins, produced as mixtures of either related or unrelated substances, have been extracted from cultures of many different bacterial and fungal pathogens. In fact, the ability to produce one or more toxins and to use them as chemical weapons may be the decisive factor in the virulence of plant pathogens, and perhaps even their pathogenicity (Rudolph, 1990). A limited number of toxins, the so-called host-selective toxins, affect only plants of one specific genotype susceptible to the pathogen. At low concentrations, these toxins reproduce the symptoms of natural infections. By far, however, the majority of phytotoxins are nonselective and do not reproduce the patterns of resistance and susceptibility of

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the host to the pathogen. These include all the bacterial phytotoxins described thus far and the majority of fungal toxins. Because these biologically active substances are broad-spectrum toxins that can affect many economically important crops, their total impact on agriculture has serious repercussions (Graniti, 1991).

Botrytis cinerea, the causative agent of the devastating gray mold disease in many economically important crops, produces characteristic metabolites with a botryane skeleton (Durán-Patrón et al., 1999). The role of these metabolites in the physiology of the fungus was hitherto unknown. Nevertheless, in previous work (Collado et al., 1996; Rebordinos et al., 1996; Durán-Patrón et al., 1997) we reported the phytotoxic effects of some of these compounds when they were assayed *in vitro* on leaf discs of *Nicotiana tabacum.*

Recently, the fungal metabolite botrydial was detected for the first time in plant tissues infected with isolates of *B. cinerea* (Deighton et al., 2001). The phytotoxin was produced in the soft rot regions of the infection. While it has not been ruled out that other toxins with a botryane skeleton may also be present in the tissues, the methodology of this particular study was applicable only to botrydial.

We have long felt that a greater understanding of the role of this toxin and its related compounds in the processes leading to disease onset and lesion expansion would be gained if the action mechanisms of the toxins involved were known. This paper describes experiments designed to determine the role of these compounds in the pathogenicity of the fungus *B. cinerea*. Phytotoxic assays with metabolites with the botryane skeleton (Figure 1) extracted from cultures of *B. cinerea* were studied. These assays were performed both *in vitro* and *in vivo* on leaves of *Phaseolus vulgaris*, as well as on different fruits. The effect of light on the action mechanism of botrydial was also studied.

METHODS AND MATERIALS

General. The strain *B. cinerea* Pers.: Fr. 2100 employed in this work was obtained from the Colección Española de Cultivos Tipos (CECT), Facultad de Biología, Universidad de Valencia, Spain. The procedures used for the isolation and purification of compounds **1–6** (Figure 1) have been described previously (Durán-Patrón et al., 1999). Pure metabolites were dissolved in 40% acetone (v/v) in 100 ml of water containing 10 μl of Tween 80. Solutions of different concentrations, ranging from 10 to 1000 ppm, were then prepared. Controls consisted of mixtures of aqueous acetone that had been used for dissolving the products.

Plant Material and Methods of Culture. Seeds of *Phaseolus vulgaris* genotypes N90598 (susceptible to *B. cinerea*) and N90563 Huron (resistant to *B. cinerea*) (Kelly et al., 1994) were obtained from J. D. Kelly, Michigan State University, East Lansing, Michigan, USA, and were grown in a standardized and quality controlled compost/sand mixture in individual pots in a greenhouse for two or three weeks at 25°C.

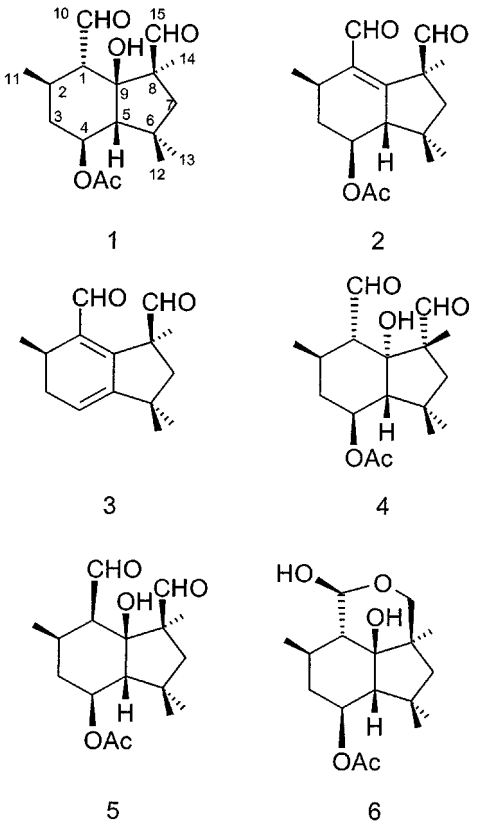

FIG. 1. Structures of compounds **1–6**.

Leaf Disc Assays. Leaves of *Phaseolus vulgaris* plants were sterilized with 10% bleach (v/v) for 3 min., washed four times with sterile water, dried between filter paper, cut into circles measuring 1 cm diam., and placed in Petri dishes (10 circles per dish) lined with Whatman paper wetted with sterile water. Each circle received a 10 μl droplet of pure compound solutions. The dishes were then incubated at 25–28°C in natural sunlight. Assays were carried out in duplicate.

The frequency of infection was ascertained by comparing the number of affected circles to the total number treated. The size of the damaged area was obtained by determining the affected surface area as compared with the total surface area, expressed in square millimeters.

Whole Plant Assays. Attached leaves were sterilized with an aqueous solution of 10% of ethanol (v/v), washed with sterile water, and dried between filter paper. In general, two *Phaseolus* plants were used per experiment. Five leaves per plant

were treated with a solution of the compound to be tested. Each leaf received five drops (10 μl) of control and five drops (10 μl) of the test solution. Two experiments were carried out for each compound and concentration. The plants were incubated at 25–28°C in natural sunlight for a minimum photoperiod of 12 hr. The diameter of the drops was identical in each case, and the total surface area of the drops was considered the treated area. The total treated area was considered to be 100%, and the affected area was calculated in relation to treated area.

Fruit Assays. Fruits (*Capsicum annuum*, *Licopersicom esculentum*, *Vitis vinifera*, *Phaseolus vulgaris*, *Fragaria vesca*, *Actinidia chinensis planchon*) were sterilized as described above and placed in Petri dishes lined with Whatman paper wetted with sterile water. Botrydial was dissolved to different concentrations (50–250 ppm) and was placed in 10 μl drops on the fruit surface. The treated fruit, five pieces per assay, was incubated at 25–28°C in natural sunlight. Two experiments were conducted for each type of fruit.

Inoculation Experiments. Sporulating fungal colonies of *B. cinerea* Pers.: Fr 2100 were first rinsed with an inoculating medium consisting of Gamborg's B5 obtained from Sigma Chemical CO (3.2 g/liter), sucrose (10 mM), and potassium phosphate buffer (0.01M, 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 approx. 10^5 conidia/ml. Five l-microliter droplets of conidial suspension were placed onto the leaf surfaces, and the leaves or plants were incubated in a chamber at 25°C in natural sunlight and with saturated air humidity.

The sunlight radiation was measured with the aid of a pyranometer (silicon cell pyranometer, SKS1110). An average total radiation of 514 W/m^2 and 360 W/m^2 was achieved, at plant level, for sunny and cloudy days, respectively.

RESULTS AND DISCUSSION

In an initial survey, we compared the activities of the phytotoxic metabolites produced by *B. cinerea in vitro* by incubating aliquots of solutions of the metabolites on leaf discs of *P. vulgaris* (genotype N90598) at concentrations of 1000, 500, 250, and 100 ppm. The more active metabolites were tested at concentrations under 100 ppm. The leaf circles were examined each day. The maximum phytotoxic effect was reached at different times for each metabolite, and remained constant thereafter. By the seventh day, the maximum effect for all metabolites had been reached (see Table 1; note that only those concentrations that gave a positive result are listed). Botryendial (**2**), botrydienal (**3**), and 8,9-epibotrydial (**4**) were highly phytotoxic; they produced chlorotic lesions at concentrations of 25, 25, and 50 ppm, respectively. Botrydial (**1**) had the highest level of activity; it caused a chlorotic effect even at a physiological concentration (1 ppm). Metabolites **5** and **6** were moderately active at a minimum concentration of 250 ppm.

TABLE 1. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY METABOLITES OF *B. cinerea* ON *Phaseolus vulgaris* LEAF DISCS

Metabolite concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]
Botrydial (**1**)		
1000	100	100
500	100	100
250	100	90
100	100	76
50	100	40
10	80	15
1	30	<5
Botryendial (**2**)		
1000	100	100
500	100	68
250	100	30
100	100	15
50	100	13
25	100	9
Botrydienal (**3**)		
1000	100	100
500	100	73
250	100	45
100	100	34
50	100	20
25	100	12
8,9-Epibotrydial (**4**)		
1000	100	100
500	100	38
250	100	20
100	100	15
50	80	10
1-Epibotrydial (**5**)		
1000	100	20
500	100	16
250	90	11
Dihydrobotrydial (**6**)		
1000	100	24
500	100	20
250	100	14

[a] Arithmetic mean of the results from two independent experiments with 10 circles for each concentration in each experiment (standard deviation <3%).

[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

TABLE 2. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY METABOLITES OF *B. cinerea* ON *Phaseolus vulgaris* PLANTS

Metabolite concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]
Botryendial (**2**)		
1000	100	100
500	100	80
250	100	70
100	100	35
50	100	<5
25	50	<5
Botrydienal (**3**)		
1000	100	100
500	100	90
250	100	75
100	100	40
50	70	15
25	50	5
8,9-Epibotrydial (**4**)		
1000	100	100
500	100	95
250	100	90
100	100	20
50	90	5
1-Epibotrydial (**5**)		
1000	100	60
500	80	35
250	60	25
Dihydrobotrydial (**6**)		
1000	100	45
500	100	15
250	80	<5

[a] Arithmetic mean of the results from two independent experiments with 10 leaves for each concentration in each experiment (standard deviation <3%).
[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

Compounds **1**–**6** were tested in whole-plant assays with *Phaseolus vulgaris*, genotype N90598. Although the correlation between *in vitro* and *in vivo* experiments was generally good, the phytotoxic effect was more rapid *in vivo*, being noticeable after only a few hours (Tables 2 and 3). It is worth noting that all compounds assayed caused severe chlorosis and cell collapse. Botrydial (**1**) was active from 1 ppm and reproduced symptoms of plant disease in 70% of the treatments.

The results described above confirm those previously reported concerning the structure–activity relationships of compounds with the botryane skeleton

TABLE 3. FREQUENCY AND SEVERITY OF LESIONS CAUSED BY BOTRYDIAL (**1**) ON *Phaseolus vulgaris* PLANTS

Botrydial concentration (ppm)	Frequency of lesion (%)[a]	Severity of lesion (%)[b]	Time (hr)
1000	100	100	0.5
500	100	100	1
250	100	100	2
100	100	100	2
50	100	40	4
40	100	35	4
30	100	30	6
20	90	15	6
10	80	6	120
1	70	5	120

[a] Arithmetic mean of the results from two independent experiments with 10 leaves for each concentration in each experiment (standard deviation <3%).
[b] Calculated as the surface affected compared to the total surface expressed in mm^2.

(Durán-Patrón et al., 1999). The biological activities of these compounds are related to the oxidation state of the C-10 and C-15 carbon atoms, as well as to the presence of a hydroxyl group on the C-9 carbon atom.

It was also observed that both sunlight radiation intensity and exposure period caused a significant difference in the time necessary for the lesions to appear. Botrydial (**1**), the most active compound, was shown to act rapidly and cause chlorotic lesions in the whole area of application at all the different concentrations assayed when the plant was exposed to bright sun light (514 W/m^2 average radiation; Table 3). In contrast, when the experiment was repeated in dim light (360 W/m^2 average radiation), the same effect took twice as long to occur. At the minimum active concentrations of 10 and 1 ppm, chlorosis took place after 120 hr in 80% and 70% of the treatments, respectively. Other highly active metabolites such as botryendial (**2**) and 8,9-epibotrydial (**4**) showed phytotoxic activity at higher concentrations after 2 and 3 hr, respectively. These experiments were carried out with two genotypes of *Phaseolus vulgaris*: genotype N90598, with well-characterized susceptibility to *B. cinerea*, and genotype N90563, with characterized resistance to the fungus. The results were similar in both cases.

In order to prove the light dependence of botrydial (**1**), the compound was applied at different concentrations (10–150 ppm) to leaves of *Phaseolus vulgaris*, which were then covered with aluminum foil. The leaves were examined every 2 hr for 12 hr and after 24 hr, and no chlorosis was evident. Nevertheless, once these same leaves were exposed to light, chlorosis was observed within 1–2 hr.

In another experiment, botrydial (**1**) was applied to the leaves of *P. vulgaris* at different concentrations, after which the plant was put in bright sunlight (514 W/m^2 average radiation). Once the lesion appeared, the leaf was covered with aluminum foil and the extent of the lesion was noted after 2, 4, 6, and 24 hr. No difference could be observed between the covered lesion and that of a leaf exposed to sunlight over the same time intervals.

As *B. cinerea* is a fungus that can affect all parts of the plant, including the fruit, we decided to observe the effect of this toxin on several fruits. When botrydial (**1**) was assayed on the surface of a fruit, a lesion in the fruit skin was observed in five of the six fruits studied. The exception was green pepper.

Finally, the effect of botrydial (**1**) on fungal penetration was studied on attached leaves of *Phaseolus vulgaris*. Botrydial was applied at different concentrations to the leaves, which were then inoculated with *B. cinerea*. The results showed that botrydial facilitates fungal penetration and colonization of plant tissue. In a typical experiment in which a 250 ppm solution of botrydial was used, 48 hr after the leaves had been inoculated with *B. cinerea*, the fungus had already started to grow, producing a lesion 43 mm in diameter. In contrast, in the inoculation experiment without botrydial, fungal penetration was slower, being visible only after 72 hr, and the diameter of the lesion was only 37 mm.

In conclusion, we have found that *B. cinerea* synthesizes a number of metabolites that reproduce some of the symptoms of the disease caused by this organism. As a result of our experiments, we have also obtained evidence that the action mechanism of the phytotoxin botrydial is light dependent.

Exogenous applications of the most active and abundant metabolite, botrydial (**1**), facilitate fungal penetration and colonization of plant tissue. These results, together with the recent discovery that botrydial is produced in infected plants (Deighton et al., 2001), suggest the involvement of this compound as a toxin in the pathogenesis of *Botrytis cinerea.* Furthermore, the fact that botrydial affects different crops and genotypes seems to indicate that this toxin and its related compounds are nonhost specific toxins that increase the extent of disease, and most likely are virulence factors. Further work is in progress to investigate the mechanistic aspects as well as the scope of the light dependent plant-toxin reaction.

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