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Formation of phytoalexins within and outside lesions of Botrytis cinerea in French bean leaves

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Abstract

Rapidly spreading lesions and lesions restricted in size developed in primary leaves of French bean (*Phaseolus vulgaris*) in response to infection by *Botrytis cinera* isolates BC-1 and BC-5, respectively. These isolates caused similar differential lesions in leaves of cucumber, flax, lettuce and tomato. To determine whether phytoalexin accumulation was correlated with the resistant reaction in bean leaves, accumulation of phytoalexins was examined in necrotic areas of both types of lesions and in their surrounding green tissues.

Phaseollin was the predominant phytoalexin, both inside and outside lesions, whereas phaseollidin and sometimes also phaseollinisoflavan were always present in lower concentrations. Phaseollin accumulated earlier and to higher levels within and around lesions of isolate BC-5 than of isolate BC-1. Relatively low concentrations of phaseollin were detected in the more remote green areas, including the petiole, of leaves bearing a spreading lesion. The phaseollin metabolite, 6a-hydroxyphaseollin, was found only inside lesions and in a narrow zone around lesions of both types.

The authors consider the possibility that the differing concentrations of phytoalexins in the infected tissues are not a determining factor for the differential interactions between *B. cinerea* and bean leaves, but are rather the result of it.

Additional keywords: Phaseollin, phaseollidin, phaseollinisoflavan, 6a-hydroxyphaseollin, *Phaseolus vulgaris*.

Introduction

Rapidly spreading lesions develop in primary leaves of French bean (*Phaseolus vulgaris* L.) after inoculation with the pathogenic isolate BC-1 of *Botrytis cinerea* Pers. ex Nocca & Balb., whereas lesions formed after inoculation with the nonpathogenic isolate BC-5 remain restricted in size (Van den Heuvel, 1976). The occurrence of phytoalexins in the necrotic tissues of such lesions and an adjacent 3-mm-wide green zone, was studied by Van den Heuvel and Grootveld (1978). In most cases phaseollin, phaseollidin, phaseollinisoflavan, the phaseollin metabolite, 6a-hydroxyphaseollin, and a few unidentified antifungal compounds were found; phaseollin was predominant. The concentration of phaseollin accumulating in leaves infected by isolate BC-5 was about twice as high as that in infections produced by isolate BC-1. However, the results were not conclusive enough to assess whether phaseollin was the principal factor which limited lesion development.

This prompted us to investigate more in detail the concentration of phytoalexins within and outside lesions of both types. García-Arenal et al. (1978) suggested that phytoalexins were involved in the limitation of *B. cinerea* lesions on bean hypocotyls.

Wasfy et al. (1978), however, explained differences in pathogenicity between *B. cinerea* isolates on bean pods and fruits of strawberry and apricot in terms of differences in activities of enzymes produced by the isolates. We, therefore, inoculated leaves of some plants known as hosts of *B. cinerea*, with isolates BC-1 and BC-5, to determine whether factors other than phytoalexins may be involved in lesion limitation.

Materials and methods

Fungal isolates and inoculation of bean leaves. The *B. cinerea* isolates BC-1 (causing spreading lesions) and BC-5 (causing lesions restricted in size) were the same as described previously (Van den Heuvel, 1976). Methods for growing these fungi, for inoculating the primary leaves of *Phaseolus vulgaris* 'Dubbele Witte zonder draad' and for incubating the plants were similar to those reported by Van den Heuvel and Grootveld (1978). Leaves of other plants were inoculated with inoculum plugs taken from the edge of 3-day-old colonies of *B. cinerea* isolates grown on PDA plates.

Preparation of tissue samples and extraction of phytoalexins. At various times, from 1 to 3 days after inoculation, infected bean leaf material was collected. Lesions and surrounding green tissues were removed separately from the leaves. The extraction of phytoalexins from this material was as described by Van den Heuvel and Grootveld (1978).

Detection, identification and quantitative analysis of phytoalexins and 6a-hydroxyphaseollin. Detection, identification and quantitation of phytoalexins and 6a-hydroxyphaseollin were done by the procedures of Van den Heuvel and Glazener (1975) and Van den Heuvel and Grootveld (1978).

Results

Phytoalexins. The concentrations of phytoalexins and of the phaseollin metabolite, 6a-hydroxyphaseollin, within lesions and in two adjacent zones of green tissue, 3 and 10 mm wide, respectively, were determined at 12, 24 or 48 h after inoculation with *B. cinerea* isolate BC-1 or BC-5. Since at 12 h after inoculation no lesions were yet apparent, the inoculated areas (of about 4 mm diameter) and their adjacent zones, 3 and 10 mm wide, were investigated. Results of the experiments are given in Fig. 1 and Table 1.

Phaseollin was the predominant phytoalexin. It was detected in the spreading lesions of isolate BC-1 not earlier than at 48 h after inoculation, and was found around lesions at 24 h after inoculation. It accumulated to 357 µg/g dry weight of green tissue in a 3-mm-wide zone around 2-day-old lesions. In leaves infected with isolate BC-5, phaseollin was already detected in the inoculated area at 12 h after inoculation and accumulated both within the lesions which became limited in size and in the surrounding zones of green tissue, to a maximum of 565 µg/g dry weight of tissue in a 3-mm-wide zone adjacent to 2-day-old lesions.

Fig. 1. Concentrations of phaseollin and 6a-hydroxyphaseollin within lesions and in two adjacent zones of green tissue, 3 and 10 mm wide, respectively, at 12, 24 or 48 h after inoculation with *B. cinerea* isolate BC-1 or BC-5.

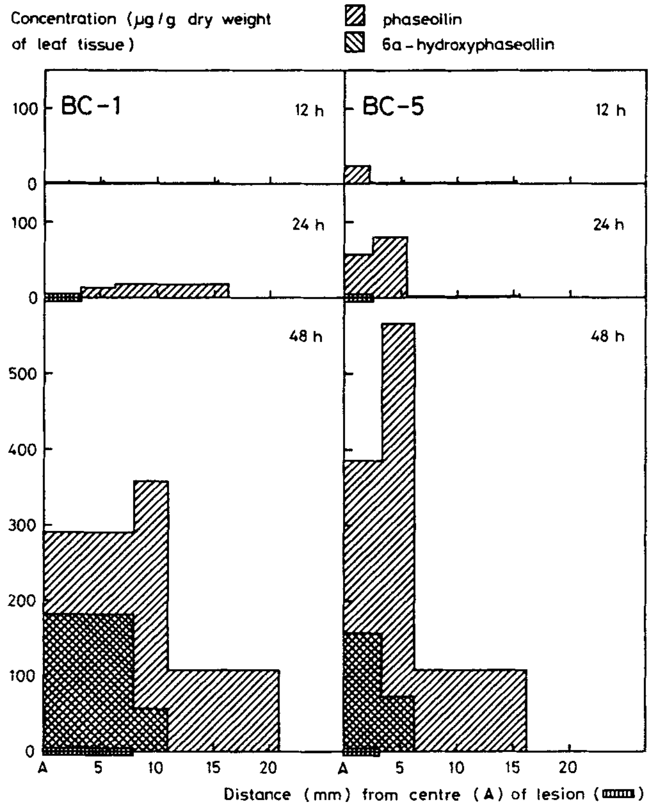

Fig. 1. Concentratie van phaseolline en 6a-hydroxyphaseolline in de lesies en in twee aangrenzende zones van groen weefsel van 3, resp. 10 mm breedte, op 12, 24 en 48 uur na inoculatie met isolaat BC-1 en BC-5 van B. cinerea.

6a-Hydroxyphaseollin was found with certainty only within 2-day-old lesions of both types and in their adjacent 3-mm-wide zone of green tissue. The concentration of 6a-hydroxyphaseollin within the lesions was always higher than in the adjacent green tissues. Phaseollidin and phaseollinisoflavan were detected only in leaves with 2-day-old lesions. The highest concentrations of these phytoalexins were found in the 3-mm-wide zone of green tissue around the lesions.

In these and in subsequent experiments, none of the other known bean phytoalexins, kievitone and 2′-methoxy-phaseollinisoflavan, were detected. In contrast, García-Arenal et al. (1978) found high concentrations of kievitone in bean hypocotyls infected by *B. cinerea*. Other antifungal compounds, however, similar to those reported previously (Van den Heuvel and Grootveld, 1978) were found in some of the investigated tissues. The compound designated B-B appeared to be identical with a sample of genistein, kindly provided by Dr H. Grisebach, Albert Ludwigs University, Freiburg i. Br., FRG, as demonstrated by R_f value (0.38) following co-chromatography on thin-

Table 1. Concentrations of phaseollidin (PHD) and phaseollinisoflavan (PIF) within and around 2-day-old lesions of isolate BC-1 or BC-5 in primary bean leaves.

Leaf tissue	Concentration (µg/g dry weight of leaf tissue)			
	Isolate BC-1		Isolate BC-5	
	PHD	PIF	PHD	PIF
Within lesions	56	tr[2]	tr	tr
In first, 3 mm wide, zone of green tissue	113	tr	101	65
In second, 10 mm wide, zone of green tissue	0[1]	0	tr	tr

[1] 0 = not detected; detection limit ca. 5 µg/g dry weight of tissue.
[2] tr = trace amounts, i.e. between ca. 5 and 10 µg/g dry weight of tissue.

Tabel 1. Concentratie van phaseollidine (PHD) en phaseollineïsoflavaan (PIF) in en rondom 2 dagen oude lesies van isolaat BC-1 en BC-5 in primaire bonebladeren.

layer silica gel plates, reaction with spray reagents, UV spectrum and mass spectrum. This mass spectrum was in good agreement with that of genistein reported by Ingham (1976). Although no quantitative studies of these compounds were made, the highest concentrations of genistein were apparent in a narrow zone of green tissue adjacent to 1- and 2-day-old spreading lesions.

The concentrations of phytoalexins are not necessarily evenly distributed in the zones investigated. Of particular interest is the concentration of phaseollin and other phytoalexins in close proximity to the tips of the advancing fungal hyphae, i.e. at the lesion margin. Therefore, the concentrations of phytoalexins were investigated in a 1-mm-wide zone of green tissue adjacent to the necrotic cells of the lesion margin. These assays were made 2 days after inoculation of leaves when accumulation of phytoalexins is known to have reached its peak (Table 2). In this 1 mm zone the concentrations of phaseollin and phaseollidin were slightly higher (for isolate BC-5) or considerably less

Table 2. Concentrations of phytoalexins and 6a-hydroxyphaseollin in a 1-mm-wide zone of green tissue around 2-day-old lesions of *B. cinerea* in primary bean leaves.

B. cinerea isolate	Concentration (µg/g dry weight of leaf tissue)			
	PHA[1]	PHD	PIF	HYD
BC-1	129	39	0[2]	52
BC-5	627	118	0	71

[1] PHA = phaseollin; PHD = phaseollidin; PIF = phaseollinisoflavan; HYD = 6a-hydroxyphaseollin.
[2] 0 = not detected; detection limit ca. 5 µg/g dry weight of tissue.

Tabel 2. Concentratie van fytoalexinen en 6a-hydroxyphaseolline in een 1 mm brede zone van groen weefsel rondom 2 dagen oude lesies van B. cinerea in primaire bonebladeren.

(for isolate BC-1) than the concentrations in the 3 mm zone (see Fig. 1 and Table 1).

As results indicated that phaseollin and also traces of phaseollidin and phaseolliniso-flavan accumulated in green tissues up to 13 mm from the lesion margin, also more remote green areas were investigated for the presence of phytoalexins. Leaves were inoculated with isolate BC-1 or BC-5 on the middle vein, to stimulate a possible spread of phytoalexins or phytoalexin elicitors throughout the leaf. As isolate BC-5, when inoculated on the middle vein, in general failed to produce lesions, only leaves in-oculated with isolate BC-1 were examined. Concentrations of phytoalexins were in-vestigated in 3-day-old spreading lesions, in a 3-mm-wide zone of green tissue adjacent to the lesions and in three surrounding 10-mm-wide zones of green tissue, and in the petiole. The two outermost zones were divided into a top and a basal half (Fig. 2).

Compared to other experiments, concentrations of phaseollin in the lesions and in the adjacent 3 mm zone of green tissue were relatively low. Phaseollin was, however, present in all areas, except that the top part of the outermost zone of green tissue was not examined due to insufficient amounts of tissue. Phaseollidin and phaseolliniso-flavan were present in low concentrations (13 to 21 µg/g dry weight of tissue) in lesions and in the two innermost green zones, but were not detected in the more remote green

Fig. 2. Concentrations of phaseollin and 6a-hydroxyphaseollin within 3-day-old lesions of *B. cinerea* isolate BC-1, and in the green areas of the infected leaves sectioned into 3 and 10 mm zones and the petiole according to the scheme presented. T = top, B = basal half of zones of green tissue.

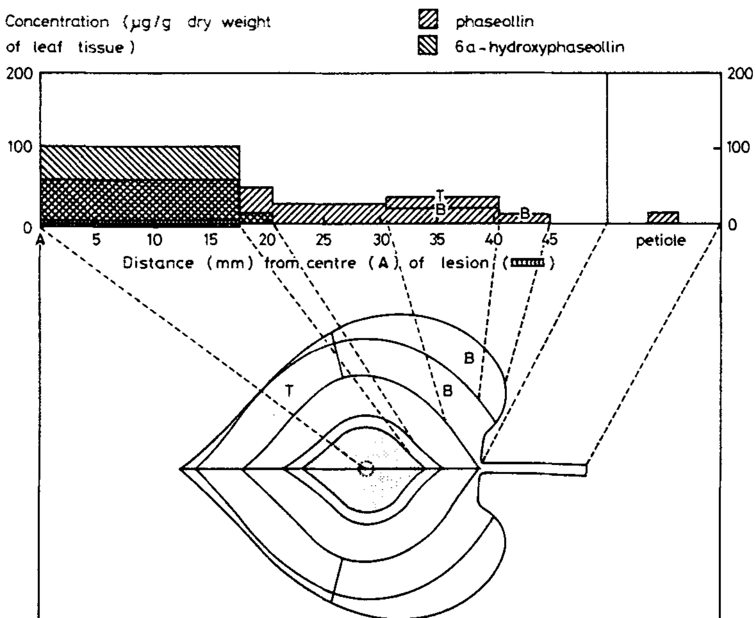

Fig. 2. Concentratie van phaseolline en 6a-hydroxyphaseolline in 3 dagen oude lesies van B. cinerea isolaat BC-1 en in de groene delen van de geïnfecteerde bladeren verdeeld in 3 en 10 mm brede zones en de bladsteel volgens het aangegeven schema. T = top, B = basale helft van zones van groen weefsel.

Table 3. Symptom development in leaves of cucumber, flax, lettuce and tomato after inoculation with *B. cinerea* isolates BC-1 and BC-5.

Plant species	Symptoms
Cucumber 'Bitspot'	BC-1: Lesions visible 1 day after inoculation and expanding very rapidly, until leaves collapsed BC-5: Necrosis visible 1 to 2 days after inoculation, developing into dry lesions of limited size, extending slightly beyond the edge of the inoculum plug. In general, lesions had a brown margin, often surrounded by a wide chlorotic halo
Flax (cultivar unknown)	BC-1: Similar as on cucumber BC-5: Similar as on cucumber. Lesions surrounded by a narrow chlorotic halo
Lettuce 'Renate'	BC-1: No lesions visible until about 7 days after inoculation. Then lesions expanding very rapidly, until leaves collapsed BC-5: Similar as on cucumber. Lesions without a chlorotic halo
Tomato 'Moneymaker'	BC-1: Similar as on lettuce BC-5: Similar as on cucumber. Lesions without a chlorotic halo

Tabel 3. Symptomen in bladeren van komkommer, vlas, sla en tomaat na inoculatie met isolaten BC-1 en BC-5 van B. cinerea.

areas. 6a-Hydroxyphaseollin was detected in lesions and in the adjacent 3-mm-wide zone of green tissue only.

Inoculation of leaves of other plants. Leaves of some plant species known as hosts of *B. cinerea* were inoculated with isolates BC-1 and BC-5. Each isolate was inoculated onto one or two leaves of each of ten 1-week-old flax plants, or onto three leaves of 3-to-6-week old plants of cucumber, lettuce and tomato (five to nine plants per inoculation). Symptom development was followed during 11 days after inoculation (Table 3). The isolates BC-1 and BC-5 caused spreading lesions and lesions restricted in size, respectively, comparable to those formed in primary leaves of *Phaseolus vulgaris*.

Discussion

Although the concentrations of phytoalexins and 6a-hydroxyphaseollin in the infected primary bean leaves varied from experiment to experiment, the results were, in general, in good agreement with those obtained previously (Van den Heuvel and Grootveld, 1978).

The highest concentrations of phaseollin and other phytoalexins occurred, 2 days after inoculation, in a narrow zone of green tissue adjacent to the lesions. On the assumption that the antifungal activity of phaseollidin is additive to that of phaseollin, and that isolate BC-1, as well as isolate BC-5, is equally sensitive to both phytoalexins, then calculations based on previous data of Van den Heuvel (1976) may be made. It is estimated that the highest concentrations of combined phytoalexins found in tissues will cause about 60 to 77% and about 90% inhibition of mycelial growth of isolates BC-1 and BC-5, respectively, in vitro. A much higher concentration, probably six-fold, of phaseollin (1.25 mM or approximately 4000 μg/g dry weight of tissue) would be needed to give 99% inhibition of isolate BC-5. There is no evidence that such a concentration will occur in the infected tissues, even at very close proximity to the hyphal tips at the lesion margin. As long as no methods are available to investigate the phytoalexin concentrations at the cellular level and to determine the sensitivity of isolate BC-5 to the phytoalexins in vivo, no firm conclusion can be made as to whether phytoalexins are the principal factor that limits development of lesions of isolate BC-5.

The results of the inoculation experiments on leaves of other plants suggest that nonpathogenicity of isolate BC-5 is due to an inability to overcome a more common kind of barrier not restricted to bean. The formation of such a barrier may be induced by isolate BC-5. The occurrence of a brown margin around the limited lesions, possibly due to the action of phenol oxidases, may indicate an inhibitory mechanism. Indeed, Wasfy et al. (1978) found that a weaker pathogenicity of *B. cinerea* was correlated with a higher activity of phenol oxidase and pectin methyl esterase, together with a lower activity of amylase, maltase, catalase and poly methyl galacturonase.

If it could be demonstrated that an inhibitory factor other than phytoalexins is operating in the delimitation of lesions of isolate BC-5, then the formation of phytoalexins in bean leaves infected by *B. cinerea* could be regarded as merely an accompanying effect of the infection. In that case, the differing concentrations of phytoalexins are not the cause but rather the result of the differential interactions between *B. cinerea* and bean leaves. During the retarded growth of isolate BC-5 in the leaf, the green cells adjacent to the lesion undergo a relatively slow process of necrotization and are probably longer affected to produce phytoalexins than are cells adjacent to the rapidly spreading lesions of isolate BC-1. This is one explanation for the different accumulation of phytoalexins. A similar mechanism has been suggested to contribute to the different rate of phytoalexin production in broad bean tissues infected by *B. fabae* or *B. cinerea* (Hargreaves et al., 1977).

Although the occurrence of phaseollin in the more remote green areas of infected leaves may be the result of translocation of either phaseollin or phaseollin elicitor(s) (e.g. such as described by Dixon and Fuller, 1977) from the lesion area to other regions of the leaf, we favour the latter possibility. Phaseollin does not seem to be translocated easily, at least in hypocotyls infected by *Colletotrichum lindemuthianum* (Bailey and Deverall, 1971; Rahe, 1973; Rathmell, 1973). Moreover, in our studies, 6a-hydroxyphaseollin, a compound more polar than phaseollin, was never detected at a distance greater than 3 mm from the lesion where it was produced.

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Samenvatting

Vorming van fytoalexinen in en buiten lesies van Botrytis cinerea in bonebladeren

Na infectie van primaire bladeren van de boon (*Phaseolus vulgaris*) met isolaten BC-1 en BC-5 van *Botrytis cinerea* ontstaan zich snel uitbreidende lesies, resp. lesies van beperkte omvang. Het necrotische weefsel van beide typen lesies en de omringende groene bladgedeelten werden onderzocht op de aanwezigheid van fytoalexinen, met het doel een mogelijke correlatie te vinden tussen de resistente reactie en accumulatie van fytoalexinen.

Phaseolline was het meest voorkomende fytoalexine, zowel in als buiten de lesies, terwijl phaseollidine en soms ook phaseollineïsoflavaan in lagere concentraties aanwezig waren (Fig. 1 en 2 en Tabel 1 en 2). Phaseolline accumuleerde eerder en tot een hoger niveau in en rondom lesies van isolaat BC-5 dan van isolaat BC-1 (Fig. 1 en Tabel 2). In bladeren met een zich uitbreidende lesie werden betrekkelijk lage concentraties van phaseolline aangetoond in de groene delen wat verder van de lesie vandaan en in de bladsteel (Fig. 2). Het omzettingsprodukt van phaseolline, nl. 6a-hydroxyphaseolline, werd alleen aangetroffen in de lesies en in een smalle zone van groen weefsel rondom beide typen lesies (Fig. 1 en 2 en Tabel 2).

De auteurs wijzen op de mogelijkheid dat de verschillen in fytoalexinen-concentraties in de geïnfecteerde weefsels niet bepalend zijn voor de twee verschillende typen interacties tussen *B. cinerea* en de bonebladeren, maar veeleer het resultaat ervan zijn.

De isolaten BC-1 en BC-5 vormden ook spreidende, resp. beperkt blijvende lesies op bladeren van enkele andere planten (komkommer, vlas, sla en tomaat) (Tabel 3). Dit wijst op een onvermogen van isolaat BC-5 om een meer algemeen voorkomend afweermechanisme te overwinnen.

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