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Phytoalexin production in French bean leaves infected by Botrytis cinerea

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Abstract

French bean (*Phaseolus vulgaris*) leaves were inoculated with three pathogenic and two nonpathogenic isolates of *Botrytis cinerea* and the infected tissues, containing either spreading lesions or lesions limited in size, were investigated for the presence of phytoalexins. 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 the nonpathogenic isolate BC-5 was about twice as high as that in infections produced by pathogenic isolates. In contrast, leaves infected by the nonpathogenic isolate BC-6 only contained low concentrations of phaseollin.

Our data do not provide strong evidence that phaseollin is the principal factor that limits lesion development.

Introduction

Numerous studies have shown that fungal infections of French bean (*Phaseolus vulgaris*) plants may induce the production of phytoalexins in the infected tissues. At least five different compounds have been identified, viz. phaseollin, phaseollidin, phaseollinisoflavan, 2′-methoxy-phaseollinisoflavan and kievitone (VanEtten and Pueppke, 1976). In general, accumulation of high concentrations of phytoalexins appears to be associated with the occurrence of a necrotic hypersensitive reaction or with the delimitation of larger lesions (Bailey, 1974; Bailey and Ingham, 1971; Elliston et al., 1977; Jerome and Müller, 1958; Rahe, 1973; Smith et al., 1975).

It has been suggested that the differential pathogenicities of *Botrytis cinerea* Pers. ex Nocca & Balbis isolates to bean may depend on their varying abilities to detoxify phaseollin (Van den Heuvel, 1976). Pathogenic isolates metabolized phaseollin to the less inhibitory compound, 6a-hydroxyphaseollin, whereas nonpathogenic isolates were less able, or unable, to do so. These differential abilities might contribute to the accumulation of low versus high concentrations of phaseollin in bean tissues following infection with pathogenic or nonpathogenic isolates, respectively.

These observations and assumptions led us to investigate the production of phytoalexins in bean leaves infected by three pathogenic and two nonpathogenic isolates of *B. cinerea*.

Materials and methods

Fungal isolates and inoculation of bean leaves. The five isolates of *B. cinerea* used in this study were the same as described earlier (Van den Heuvel, 1976).

The fungi were grown, either as shake or still cultures on a modified Richards' solution with 2% glucose as the sole carbon source. After 3 to 4 days of incubation at 23°C the mycelium was filtered from the cultures and resuspended in fresh Richards' medium. Small plugs (about 4 mm diameter) of wet mycelium were placed on the adaxial surface of primary leaves of 11- to 13-day-old bean plants (*Phaseolus vulgaris* L. 'Dubbele Witte z. dr.') grown in the greenhouse at 22°–27°C. In each experiment about 85–100 plants were inoculated with one to five mycelial plugs on each primary leaf. The inoculated plants were kept at 17°–20°C in transparent humidity chambers lined with moistened filter paper to maintain a high relative humidity. The pathogenic isolates (isolates BC-1, BC-3 and BC-4) caused rapidly spreading lesions whereas the nonpathogenic isolates produced lesions limited in size (isolate BC-5) or only a few pin-point lesions (isolate BC-6) (Van den Heuvel, 1976).

Preparation of tissue samples and extraction of phytoalexins. At various times, from 1 to 4 days after inoculation, infected leaf material was collected. Young, round lesions, together with an adjacent green, 3 mm wide zone, were punched from the leaves with a cork borer. Older lesions, also with an adjacent green, 3 mm wide zone, were dissected from the leaf tissues with a scalpel. The collected material was frozen in liquid nitrogen, ground with a pestle in a mortar to a powder, and subsequently lyophilized. Two hundred mg (dry weight) samples were gently shaken in 20 ml of 60% methanol for 30 min at room temperature. Insoluble material was removed by filtering the suspension through Whatman No. 50 filter paper and washing twice with 5 ml of 60% methanol. The combined filtrate was evaporated to dryness under vacuum below 40°C. The residue was taken up in 1.00 ml of 96% ethanol; 0.85 ml of this solution was transferred to a small vial and dried under a stream of nitrogen.

The residue was redissolved in 80 µl of 96% ethanol; 70 µl of this solution was applied on top of a Sephadex LH-20 column (31 × 0.6 cm) and eluted with 96% ethanol at a rate of 6 ml/h. With this procedure a complete separation between chlorophyll pigments (contained within the first 8 ml of eluate) and phytoalexins (contained within the next 12 ml of eluate) was achieved (Table 1). The latter fraction was evaporated to dryness under vacuum. The residue was taken up in 1.00 ml of 1-propanol; 0.85 ml of this solution was transferred to a small vial and dried under a stream of nitrogen. The residue was redissolved in 35 µl of 1-propanol; 25 µl of this solution was applied as a 15 mm long band on a silica gel thin-layer chromatography (TLC) plate (Merck Silica Gel 60 F_{254}, layer thickness 0.25 mm), previously scored into 35 mm wide lanes. Appropriate phytoalexins, which had been isolated and purified from bean hypocotyls infected with *Fusarium solani* f. sp. *phaseoli*, *Rhizoctonia solani* or tobacco necrosis virus, were spotted as reference compounds. The plates were developed using toluene:ethyl acetate:methanol (25:8:1) as the solvent system (Table 2).

Detection and identification of phytoalexins. Phytoalexins and other antifungal substances were detected by spraying developed chromatograms with conidia of

Table 1. Elution volumes of bean chlorophyll pigments, phytoalexins and 6a-hydroxyphaseollin from a Sephadex LH-20 column (31 × 0.6 cm) with 96% ethanol as eluant.

Compound	Elution volume (ml)[1]
Chlorophyll pigments	5– 8
Phytoalexins:	
Phaseollin	11–13
Phaseollidin	10–13
Phaseollinisoflavan	10–14
Kievitone	11–17
6a-Hydroxyphaseollin	10–13

[1] One-ml fractions of the eluate were examined for the presence of pigments and phytoalexins by thin-layer chromatography (Merck Silica Gel 60 F_{254}).

Tabel 1. Elutievolumes van chlorofylpigmenten, fytoalexinen en 6a-hydroxyphaseolline uit boon, na chromatografie over een kolom van Sephadex LH-20 (31 × 0.6 cm) met 96%-ethanol als eluens.

Table 2. R_f values of some bean phytoalexins and 6a-hydroxyphaseollin when subjected to thin-layer chromatography (Merck Silica Gel 60 F_{254}) with toluene : ethyl acetate : methanol (25 : 8 : 1) as the solvent system.

Compound	R_f value
Phaseollin	0.71
Phaseollidin	0.60
Phaseollinisoflavan	0.57
Kievitone	0.26
6a-Hydroxyphaseollin	0.47

Tabel 2. R_f-waarden van enkele fytoalexinen uit boon en 6a-hydroxyphaseolline, na dunnelaag-chromatografie (Merck Silica Gel 60 F_{254}) met tolueen:ethylacetaat:methanol (25:8:1) als loop-systeem.

Cladosporium cucumerinum suspended in Czapek-Dox solution and by incubating these plates for 3 to 4 days under humid conditions. Antifungal compounds were recognized as white or light green spots or bands of silica gel where growth of the dark green fungus had been inhibited.

Identification of phytoalexins and of 6a-hydroxyphaseollin was accomplished by comparing R_f values with those of co-chromatographed reference compounds, reaction with spray reagents (diazotized *p*-nitroaniline, Gibbs' reagent) and UV spectrometry.

Quantitative analysis of phytoalexins and 6a-hydroxyphaseollin. Quantitation of chromatographed phytoalexins and 6a-hydroxyphaseollin was carried out by in situ densitometry as described earlier for phaseollin (Van den Heuvel and Glazener, 1975). All values obtained are averages of duplicate experiments.

In control experiments known amounts of phaseollin were added to healthy bean leaves and extracted in the same manner as from infected leaves. In these experiments

80% of added phaseollin was recovered. In this paper only actually-recovered concentrations of phytoalexins are presented.

Results

Detection of phytoalexins. The TLC plate bioassays revealed the accumulation of varying amounts of phaseollin, depending on the *B. cinerea* isolate used and on the length of the incubation period before extraction (Fig. 1). Sometimes small inhibitory bands of phaseollidin or phaseollinisoflavan were also detected.

In addition, relatively large white or light green areas of up to six as yet unidentified antifungal compounds (tentatively designated B-A, B-B, etc. through B-F) were obtained, which were not present in extracts of healthy, non-inoculated leaves. The size of these areas varied with the type of extract. Two of these compounds, viz. B-B and the blue-fluorescing B-C, gave, when dissolved in ethanol and ethanolic NaOH, UV absorption spectra very similar to those reported for genistein (Biggs, 1975) and coumestrol (Keen et al., 1972), respectively.

Fig. 1. Diagram of a TLC plate bioassay of extracts from 200 mg (dry weight) samples of bean leaves collected 1 or 3 days after inoculation with mycelium of *B. cinerea* isolates BC-4 (pathogenic) or BC-5 (nonpathogenic), or after application of Richards' medium alone (control). White and light grey bands: areas of strong and weak inhibition, respectively: PHA = phaseollin; PHD = phaseollidin; PIF = phaseollinisoflavan; B-A, B-B, etc. = unidentified inhibitory compounds.

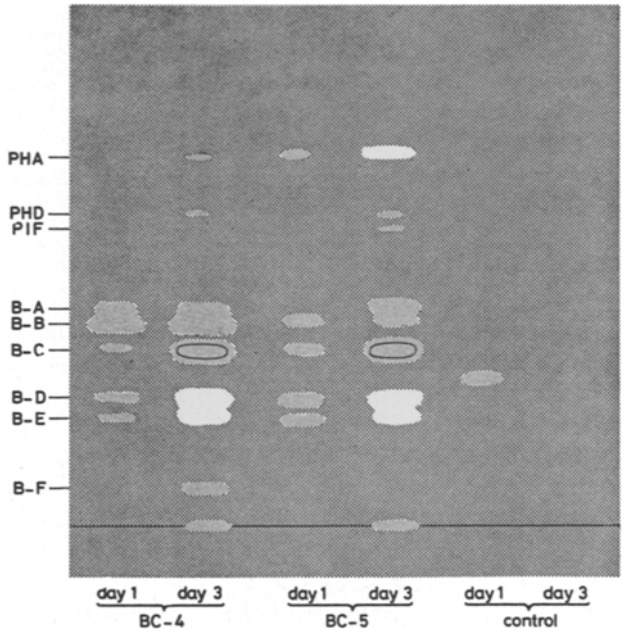

Fig. 1. Diagram van een biotoets op dunnelaag-chromatografieplaten met extracten van monsters van 200 mg (drooggewicht) van bonebladeren verzameld 1 en 3 dagen na inoculatie met mycelium van isolaat BC-4 (pathogeen) en BC-5 (niet-pathogeen) van B. cinerea, en na toediening van enkel Richards' medium (contrôle). Witte en lichtgrijze banden: plekken met sterke, resp. zwakke remming: PHA = phaseolline; PHD = phaseollidine; PIF = phaseollineïsoflavaan; B-A, B-B, etc. = niet-geïdentificeerde remstoffen.

None of the other known bean phytoalexins, kievitone and 2′-methoxyphaseollinisoflavan, were detected in infected bean leaves. No phytoalexins were found in extracts of non-inoculated leaves.

Changes in phytoalexin concentrations with time. The concentrations of phytoalexins and of the phaseollin metabolite, 6a-hydroxyphaseollin, in infected bean leaf tissues were determined at 1, 2, 3 or 4 days after inoculation. Results of these experiments are given in Figs. 2 and 3. In all samples phaseollin appeared to be the most abundant phytoalexin. In leaves infected with a pathogenic *B. cinerea* isolate, phaseollin accumulated to a maximum concentration on the second or third day after inoculation; its concentration decreased somewhat thereafter. The highest concentration found was about 300 μg/g dry weight of tissue (isolate BC-4) but, in general, the concentrations were considerably lower. 6a-Hydroxyphaseollin was always detected from the second day after inoculation, with a maximum concentration always on the third day. Relatively small amounts of phaseollidin were present in most samples. In tissues bearing lesions of isolate BC-1 low concentrations of phaseollinisoflavan were found.

In leaves infected by the nonpathogenic isolate BC-5, phaseollin reached its maximum concentration (about 320 μg phaseollin/g dry weight of tissue) on the second day after inoculation; this level was maintained during the following days. This concentration was higher than that induced by any of the pathogenic isolates. Relatively low concentrations of phaseollidin and phaseollinisoflavan were also present. 6a-Hydroxyphaseollin was detected on the second day after inoculation, its concentration increasing steadily thereafter. In leaves inoculated with the nonpathogenic isolate BC-6 low concentrations of phaseollin were found; no other phytoalexins or 6a-hydroxyphaseollin were detected.

Total numbers and total dry weights of lesions collected each day after inoculation in the experiments with isolates BC-1 (pathogenic) and BC-5 (nonpathogenic) were used to transform concentrations of phaseollin and 6a-hydroxyphaseollin into amounts per lesion (Fig. 4). The amounts of both compounds appeared to increase rapidly in expanding lesions of isolate BC-1, whereas the rate of accumulation of these compounds tended to decrease in lesions becoming limited in size, caused by isolate BC-5.

Discussion

The phaseollin content in lesion tissues of isolate BC-5 (nonpathogenic) appeared to be mostly about twice as high as that in lesions caused by the pathogenic *B. cinerea* isolates. The other phytoalexins, phaseollidin and phaseollinisoflavan, were, in general, detected in relatively low concentrations, and seem, therefore, to be of only minor importance. The formation of little phaseollin and of no other phytoalexins in response to isolate BC-6 was consistent with the occurrence of very little necrosis. Most probably other factors are responsible for the extremely limited infection by this isolate.

The concentration of phaseollin in the tissues is controlled by the balance between synthesis, metabolism and translocation of this phytoalexin. No evidence for any significant translocation of phaseollin has been obtained. The occurrence of readily

Fig. 2. Concentrations of phytoalexins and 6a-hydroxyphaseollin in lesion tissues excised from bean leaves infected by the pathogenic *B. cinerea* isolates BC-1, BC-3 and BC-4 at different times after inoculation. Vertical lines denote standard deviations.

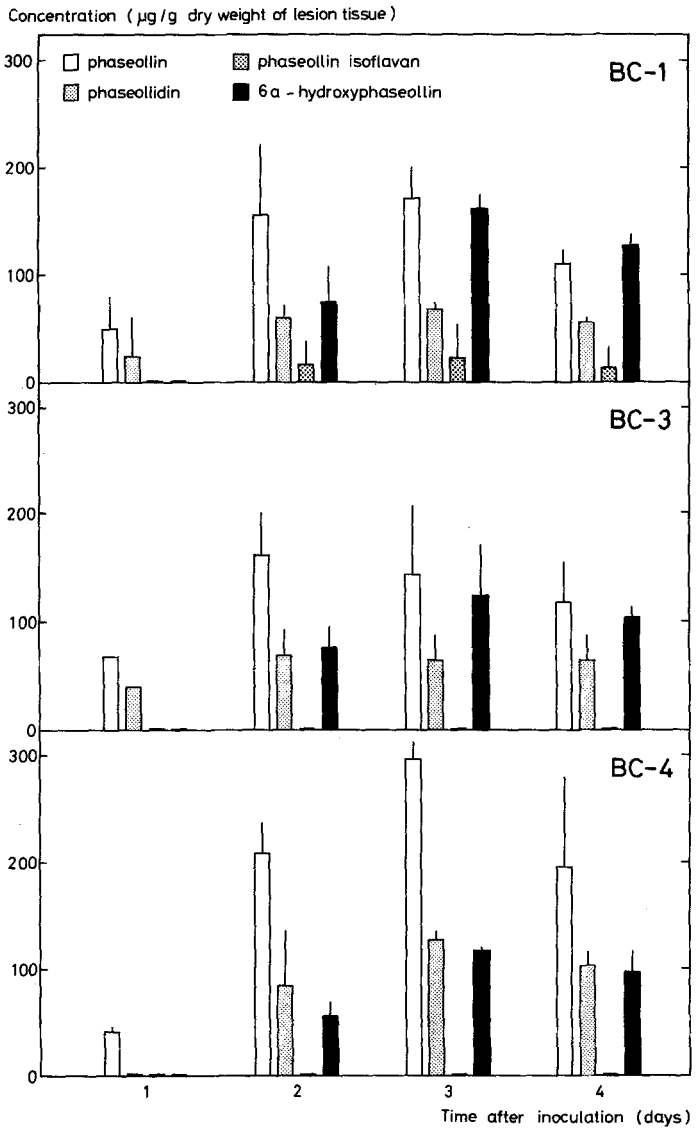

Fig. 2. Concentratie van fytoalexinen en 6a-hydroxyphaseolline in lesieweefsels van bladeren geïnfecteerd met de pathogene isolaten BC-1, BC-3 en BC-4 van B. cinerea, op verschillende tijden na inoculatie. De verticale lijntjes geven de standaardafwijkingen aan.

Fig. 3. Concentrations of phytoalexins and 6a-hydroxyphaseollin in lesion tissues excised from bean leaves infected by the nonpathogenic *B. cinerea* isolates BC-5 and BC-6 at different times after inoculation. Vertical lines denote standard deviations.

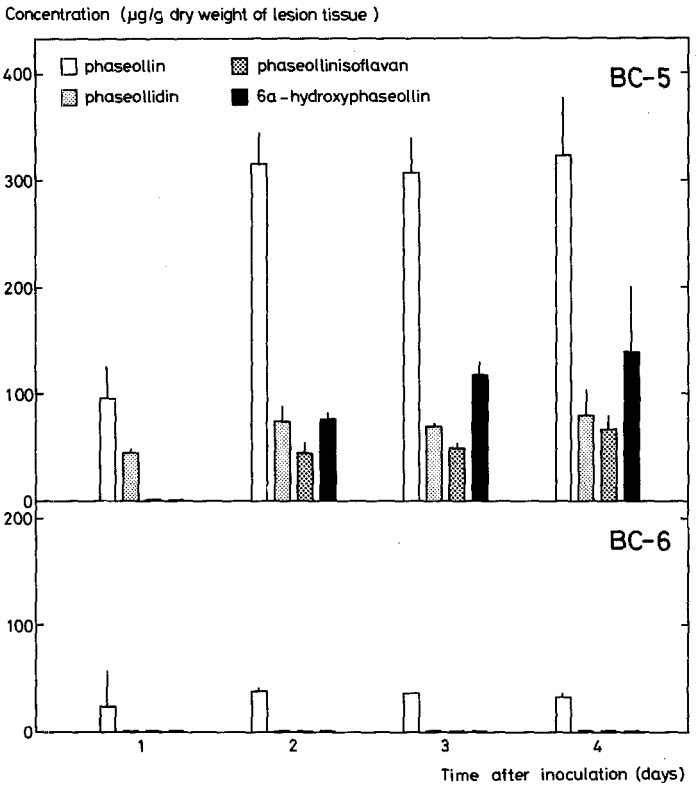

Fig. 3. Concentratie van fytoalexinen en 6a-hydroxyphaseolline in lesieweefsels van bladeren geïnfecteerd met de niet-pathogene isolaten BC-5 en BC-6 van B. cinerea, op verschillende tijden na inoculatie. De verticale lijntjes geven de standaardafwijkingen aan.

detectable concentrations of 6a-hydroxyphaseollin in lesions formed by the pathogenic isolates and isolate BC-5 points to an active metabolism of phaseollin by these isolates in the infected tissues. The different concentrations of 6a-hydroxyphaseollin relative to those of phaseollin may partly reflect the differential abilities of the pathogenic and nonpathogenic isolates to metabolize phaseollin to 6a-hydroxyphaseollin. These differential abilities, however, did not lead to very large differences in absolute phaseollin concentrations in the infected leaf tissues.

The amount of phaseollin per lesion of isolate BC-1 increased with time at a more or less constant rate, whereas the rate of accumulation of 6a-hydroxyphaseollin seemed to be exponential (Fig. 4). Growth of lesions, as measured by lesion diameter, has also been shown to occur at a constant rate (Van den Heuvel, 1976). Although older lesions are not exactly circular, their periphery may be considered to be roughly directly proportional to lesion diameter (2r), whereas their area is directly proportional to their square radius (r^2). The increase of lesion area is, therefore, also expo-

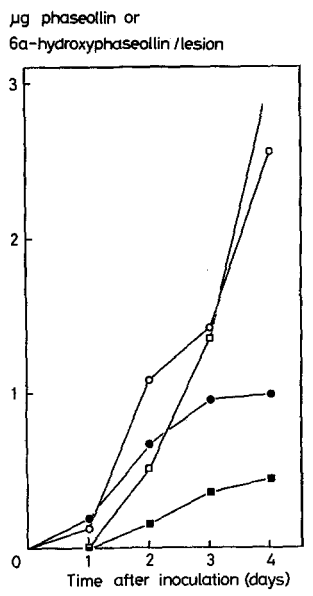

Fig. 4. Amounts of phaseollin (○, ●) and 6a-hydroxyphaseollin (□, ■) per lesion produced by the pathogenic *B. cinerea* isolate BC-1 (○, □) or by the nonpathogenic isolate BC-5 (●, ■) at different times after inoculation.

Fig. 4. Hoeveelheid phaseolline (○, ●) en 6a-hydroxyphaseolline (□, ■) per lesie gevormd door het pathogene isolaat BC-1 (○, □) of door het niet-pathogene isolaat BC-5 (●, ■) van B. cinerea, op verschillende tijden na inoculatie.

nential. This could mean that the accumulation of phaseollin is associated mainly with production of this phytoalexin in a zone immediately around the rapidly expanding lesions. In contrast, the conversion of phaseollin to 6a-hydroxyphaseollin might take place within the expanding lesions. A similar pattern of formation and metabolism of phaseollin may occur around and within lesions produced by isolates BC-3 and BC-4.

The gradually decreasing accumulation rate of phaseollin in lesion tissues infected by isolate BC-5 (Fig. 4) follows a similar pattern, as does the diameter of its lesions, which become limited in size with time (Van den Heuvel, 1976). This again may indicate that phaseollin production is located in a zone of apparently healthy cells around lesions. Here formation of 6a-hydroxyphaseollin supposedly also takes place within the necrotic tissue.

If phaseollin is evenly distributed in the examined lesion tissues, its concentrations found in spreading lesions two days after inoculation are sufficient to cause 50 to 60% inhibition of mycelial growth of pathogenic isolates under in vitro conditions. Its concentration in lesions of isolate BC-5 would then cause about 75% inhibition of growth of this isolate (Van den Heuvel, 1976). These differences do not seem to be large enough to account for the differential reaction of the leaves upon infection by the *B. cinerea* isolates. If phaseollin is located mainly in a zone around lesions, the hyphal tips may come in contact with considerably higher concentrations of phaseollin. Its concentrations are, however, apparently not high enough to inhibit strongly spreading of lesions of the pathogenic isolates. The pathogenic isolates may be better able to tolerate a rapid accumulation of phaseollin and other phytoalexins in young lesions than is the nonpathogenic isolate BC-5. Our data do not provide strong evidence that phaseollin really is the inhibitory factor in infections by isolate BC-5. The inhibitory effect of the other phytoalexins produced is presumably merely additive to the activity of phaseollin alone, as has been found by Smith et al. (1975) for

Rhizoctonia solani. It seems probable that the other, as yet unidentified, antifungal substances found in the lesion tissues, will contribute to the inhibitory potential of these tissues.

Research on the identity and fungitoxicity of these inhibitors and a detailed examination of the distribution of the phytoalexins and their metabolites within and around lesions may help to clarify the significance of phytoalexins in the infection of bean leaves by *B. cinerea*.

Samenvatting

Vorming van fytoalexinen in bonebladeren geïnfecteerd met Botrytis cinerea

Bladeren van boon (*Phaseolus vulgaris*) werden geïnoculeerd met drie pathogene en twee niet-pathogene isolaten van *Botrytis cinerea*, als gevolg waarvan zich uitbreidende lesies, resp. lesies van beperkte omvang ontstonden. Deze lesies werden samen met een omringend groen gedeelte onderzocht op de aanwezigheid van fytoalexinen. Hiertoe werden deze stoffen geëxtraheerd uit drooggevroren bladmateriaal, en gezuiverd m.b.v. kolomchromatografie over Sephadex LH-20 (Tabel 1) en dunnelaag-chromatografie (Tabel 2). Kwantitatieve analyse vond plaats d.m.v. in situ-densitometrie.

In de meeste gevallen werden phaseolline, phaseollidine, phaseollineïsoflavaan, een omzettingsprodukt van phaseolline, nl. 6a-hydroxyphaseolline, en enkele onbekende fungitoxische stoffen aangetoond (Fig. 1). Phaseolline was het meest voorkomende fytoalexine (Fig. 2 en 3). De concentratie van phaseolline in bladeren geïnfecteerd met het niet-pathogene isolaat BC-5 was ongeveer tweemaal zo hoog als in bladeren geïnfecteerd met een pathogeen isolaat (isolaat BC-1, BC-3 en BC-4). Daarentegen bevatten bladeren die met het niet-pathogene isolaat BC-6 waren geïnfecteerd slechts lage concentraties phaseolline.

De hoeveelheden phaseolline en 6a-hydroxyphaseolline, omgerekend per lesie, namen snel toe in zich uitbreidende lesies van isolaat BC-1, terwijl de toenamesnelheid van deze stoffen afnam in lesies van isolaat BC-5, die beperkt van omvang bleven (Fig. 4).

De resultaten geven geen duidelijke aanwijzingen dat phaseolline de belangrijkste remmende factor zou zijn bij het beperkt blijven van lesies.

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References

Bailey, J. A., 1974. The relationship between symptom expression and phytoalexin concentration in hypocotyls of *Phaseolus vulgaris* infected with *Colletotrichum lindemuthianum*. Physiol. Pl. Path. 4: 477–488.

Bailey, J. A. & Ingham, J. L., 1971. Phaseollin accumulation in bean (*Phaseolus vulgaris*) in response to infection by tobacco necrosis virus and the rust *Uromyces appendiculatus*. Physiol. Pl. Path. 1: 451–456.

Biggs, D. R., 1975. Post-infectional compounds from the French bean *Phaseolus vulgaris*; isolation and identification of genistein and 2′, 4′, 5, 7-tetrahydroxyisoflavone. Aust. J. Chem. 28: 1389–1392.

Elliston, J., Kuć, J., Williams, E. B. & Rahe, J. E., 1977. Relationship of phytoalexin accumulation to local and systemic protection of bean against anthracnose. Phytopath. Z. 88: 114–130.

Heuvel, J. van den, 1976. Sensitivity to, and metabolism of, phaseollin in relation to the pathogenicity of different isolates of *Botrytis cinerea* to bean (*Phaseolus vulgaris*). Neth. J. Pl. Path. 82: 153–160.

Heuvel, J. van den & Glazener, Judy A., 1975. Comparative abilities of fungi pathogenic and non-pathogenic to bean (*Phaseolus vulgaris*) to metabolize phaseollin. Neth. J. Pl. Path. 81: 125–137.

Jerome, S. M. R. & Müller, K. O., 1958. Studies on phytoalexins. II. Influence of temperature on resistance of *Phaseolus vulgaris* towards *Sclerotinia fructicola* with reference to phytoalexin output. Aust. J. biol. Sci. 11: 301–314.

Keen, N. T., Zaki, A. I. & Sims, J. J., 1972. Biosynthesis of hydroxyphaseollin and related isoflavanoids in disease-resistant soybean hypocotyls. Phytochemistry 11: 1031–1039.

Rahe, J. E., 1973. Occurrence and levels of the phytoalexin phaseollin in relation to delimitation at sites of infection of *Phaseolus vulgaris* by *Colletotrichum lindemuthianum*. Can. J. Bot. 51: 2423–2430.

Smith, D. A., VanEtten, H. D. & Bateman, D. F., 1975. Accumulation of phytoalexins in *Phaseolus vulgaris* hypocotyls following infection by *Rhizoctonia solani*. Physiol. Pl. Path. 5: 51–64.

VanEtten, H. D. & Pueppke, S. G., 1976. Isoflavonoid phytoalexins. In: J. Friend & D. R. Threlfall (Eds.), Biochemical aspects of plant-parasite relationships. Academic Press, London: 239–289.

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