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Metabolism of phaseollin by different races of Colletotrichum lindemuthianum

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Abstract

The ability of four races of the bean pathogen *Colletotrichum lindemuthianum* to metabolize the phytoalexin phaseollin in shake cultures was compared. Apart from some differences in the rate of conversion, all races metabolized the phytoalexin in the same way. Phaseollin was first converted to 6a-hydroxyphaseollin, and this product was further metabolized to 6a,7-dihydroxyphaseollin. No metabolites of the latter compound could be detected.

6a,7-Dihydroxyphaseollin was as inhibitory as phaseollin to race $\delta 11$, but was only slightly inhibitory to races $\alpha 1$, $\alpha 2$ and $\gamma 1$.

Introduction

Races β and γ of *Colletotrichum lindemuthianum* (Sacc. & Magn.) Briosi & Cav. are able to metabolize the bean phytoalexin phaseollin in vitro (Bailey, 1974). Phaseollin disappeared from shake cultures of this pathogen, and two consecutive metabolic products appeared and disappeared again. The two metabolites have been identified by Burden et al. (1974) as 6a-hydroxyphaseollin and 6a,7-dihydroxyphaseollin. Both were fungitoxic to *Cladosporium cucumerinum*. No further products of phaseollin metabolism could be detected.

Working with race $\alpha 2$ of *C. lindemuthianum* Van den Heuvel and Glazener (1975) found that also this race converted phaseollin in vitro to two metabolites. The first product was identical with 6a-hydroxyphaseollin and was as fungitoxic as phaseollin to *C. lindemuthianum*. The second metabolite, tentatively designated as compound CL_2, was characterized as a dihydroxyphaseollin, possibly 1,6a-dihydroxyphaseollin. It was suggested that different races of *C. lindemuthianum* might metabolize 6a-hydroxyphaseollin in different ways.

The purpose of the present investigation was to compare the metabolism of phaseollin by a number of different races of *C. lindemuthianum* and to further characterize the second metabolite, CL_2.

Materials and methods

Fungal isolates. The fungal isolates used in this study were races $\alpha 1$, $\alpha 2$, $\gamma 1$ and $\delta 11$ of the bean pathogen *Colletotrichum lindemuthianum*. These isolates were kindly provided by N. Hubbeling, IPO, Wageningen.

Preparation of shake cultures. The methods used for growing the fungi and preparing shake cultures were basically the same as described by Van den Heuvel and Glazener (1975), but with some modifications.

To increase sporulation, the isolates were first grown on slants of yeast-peptone-glucose agar or Mathur's medium (Mathur et al., 1950) for about 7 days at 23 °C in the dark; then conidia were transferred to plates of PDA or Mathur's medium and incubated for 6 to 10 days at 23 °C in the dark. Conidial suspensions from these cultures were used as starting material for shake cultures in Czapek Dox liquid medium.

To improve germination of conidia, flasks with suspensions were first incubated at 28 °C (Martinez Salazar and Andersen, 1957) for 24 h as still cultures, and were then incubated on a shaker at 23 °–24 °C for 3 to 7 days. When these cultures contained about 1.50 to 3.00 mg (dry weight) mycelium/ml, they were diluted with fresh Czapek Dox liquid medium to give cultures of 100 ml in 750-ml Erlenmeyer flasks, containing approximately 1.20 mg (dry weight) mycelium/ml. Purified phaseollin, dissolved in dimethylsulfoxide (DMSO), was added to these cultures to give a final phaseollin concentration of 10 μg/ml; the final DMSO concentration was 0.5%.

Extraction and quantitative analysis of phaseollin and its metabolic products. After the cultures had been shaken with phaseollin for different periods, two 8-ml samples were taken from one flask, or one 10-ml sample was taken from each of two duplicate cultures. Eight or ten ml 96% ethanol was added to each sample to halt metabolic activity and to extract phaseollin and its metabolic products from the mycelium. Further steps in the extraction procedure were similar to those described previously (Van den Heuvel and Glazener, 1975). So was the quantitative analysis of phaseollin and its metabolites, except that thin-layer chromatograms were developed in an unsaturated tank with benzene:ethyl acetate:methanol (25:8:1) as solvent system. Samples of phaseollin, 6a-hydroxyphaseollin and dihydroxyphaseollin (compound CL_2, produced by race α2) were spotted as reference compounds on thin-layer plates.

The efficiency of recovery of phaseollin after 5 min of incubation always varied from 40 to 60%. No attempts were made to determine the cause of the low efficiency. Only actually recovered percentages of phaseollin and its metabolites are presented.

Results

Metabolism of phaseollin by different races. In shake cultures of each of four different races of *C. lindemuthianum* phaseollin disappeared, while consecutively two metabolic products appeared (Fig. 1). Although there were marked differences in the rate of conversion of phaseollin by the four races, always less than 15% of the original amount of phaseollin was left in the cultures after incubation for 24 h.

Phaseollin (R_f 0.86) was always first converted to a metabolite which was identified as 6a-hydroxyphaseollin (R_f 0.62), by the reaction with diazotized *p*-nitroaniline spray reagent and UV spectrum (Van den Heuvel and Glazener, 1975). In all cases this metabolite was further converted to the same compound CL_2 (R_f0.37) as produced by race α2. Also this second metabolite disappeared from the cultures, though slowly, in particular from those of race δ11. No further metabolites were detected with this technique.

Fig. 1. Recovery of phaseollin (●) and its metabolic products, 6a-hydroxyphaseollin (○) and 6a, 7-dihydroxyphaseollin (□), from chromatographed extracts of shake cultures of four races of *Colletotrichum lindemuthianum*, incubated with 10 µg phaseollin/ml.

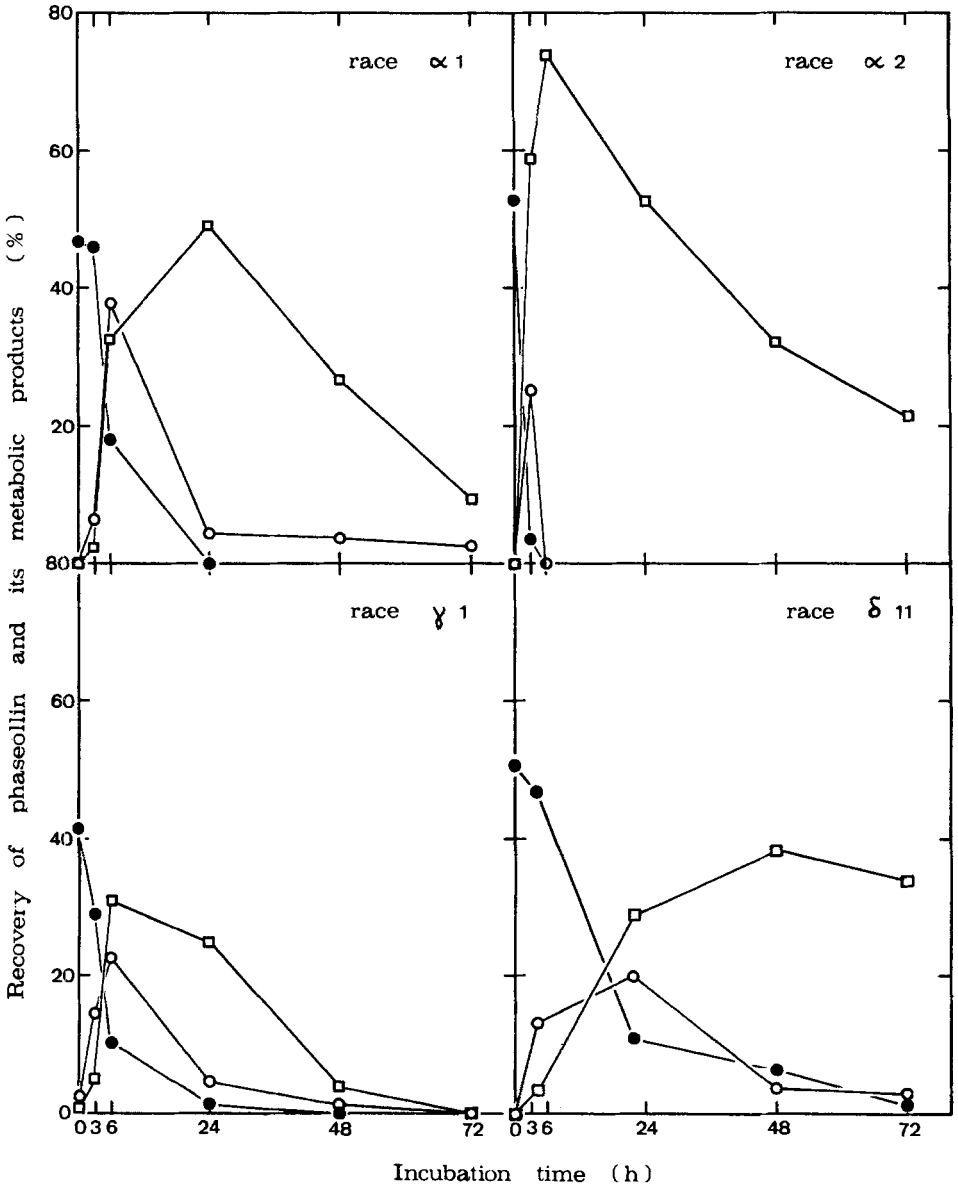

Fig. 1. Hoeveelheid teruggevonden phaseolline (●) en zijn omzettingsprodukten, 6a-hydroxyphaseolline (○) en 6a,7-dihydroxyphaseolline (□), in gechromatografeerde extracten van schudculturen van vier fysio's van Colletotrichum lindemuthianum, na incubatie met 10 µg phaseolline/ml.

Further characterization of the dihydroxyphaseollin (compound CL_2). In addition to the characteristics already described (Van den Heuvel and Glazener, 1975), the following properties of compound CL_2 as produced by race $\alpha 2$ were found.

The compound is a slightly brownish solid. It exhibits UV absorption in 96% ethanol at λ_{max} (log ε) 209 (4.37), 239 (4.42), 282 (3.96), 287 (3.97) and 310 sh (3.53) nm. Its NMR spectrum (CD_3CN) was in very good agreement with that reported by Burden at al. (1974) for 6a,7-dihydroxyphaseollin. This indicated that both compounds were identical.

Tests with Gibbs' spray reagent (Krebs et al. (1969), reagent nr. 62, with 2,6-dichloroquinone-4-chloroimide instead of 2,6-dibromoquinone-4-chloroimide) were performed on compound CL_2 and a sample of 6a,7-dihydroxyphaseollin kindly provided by J. A. Bailey, ARC Unit of Plant Growth Substances and Systemic Fungicides, Wye College. Both compounds reacted identically in a more or less positive way. After spraying, an immediate bluish green colour was obtained which turned to a more greyish shade after a few hours and later to a stable brown colour. In general, compounds which react positively obtain a stable blue or purple colour. Negatively reacting compounds such as phaseollin and 6a-hydroxyphaseollin showed only a weak brownish discolouration after spraying with Gibbs' reagent.

The results show that compound CL_2 is identical with metabolite 2 described by Burden et al. (1974) and that these metabolites must be assigned the structure of 6a,7-dihydroxyphaseollin, in spite of the apparently 'false' positive reaction with Gibbs' reagent.

The dihydroxyphaseollin produced by each of the races $\alpha 1$, $\gamma 1$ and $\delta 11$ was identical with 6a,7-dihydroxyphaseollin produced by race $\alpha 2$, as compared by R_f value following co-chromatography on thin-layer silica gel plates in three solvent systems, reaction with spray reagents, and UV spectrum.

Antifungal activity of phaseollin and 6a,7-dihydroxyphaseollin. The four races of *C. lindemuthianum* were used for comparing the inhibitory activities of phaseollin and 6a, 7-dihydroxyphaseollin. The bioassays employed were essentially the same as described previously (Van den Heuvel and VanEtten, 1973). Net radial mycelial growth was measured after 4 days of incubation.

The sensitivity of the four races to phaseollin, but not to 6a,7-dihydroxyphaseollin, was similar (Table 1). The latter compound was only slightly inhibitory to races $\alpha 1$, $\alpha 2$ and $\gamma 1$, but was as toxic as phaseollin to race $\delta 11$.

Discussion

Different races of *C. lindemuthianum* were capable of metabolizing phaseollin in vitro in a similar way. In all cases the first metabolite formed was 6a-hydroxyphaseollin, which was further converted to a compound which could be identified as 6a,7-dihydroxyphaseollin. The latter compound was further metabolized without formation of products detectable with the technique used. The metabolism of phaseollin by the races $\alpha 1$, $\alpha 2$, $\gamma 1$ and $\delta 11$, as reported here, was similar to that by races β and γ, as reported by Bailey (1974) and Burden et al. (1974). Apart from some differences in the rate of metabolism of phaseollin, no evidence was found for the hypothesis (Van den Heuvel and Glazener, 1975), that different races of *C. lindemuthianum* might meta-

Table 1. Inhibition of radial mycelial growth of four races of *Colletotrichum lindemuthianum* by phaseollin and 6a,7-dihydroxyphaseollin.

Races of *C. lindemuthianum*	Percent inhibition	
	phaseollin (0.1 mM = 32.2 μg/ml)	6a,7-dihydroxyphaseollin (0.1 mM = 35.4 μg/ml)
Race α1	38	6
Race α2	26	9
Race γ1	26	13
Race δ11	33	32

Tabel 1. Remming van de radiale myceliumgroei van vier fysio's van Colletotrichum lindemuthianum door phaseolline en 6a,7-dihydroxyphaseolline.

bolize 6a-hydroxyphaseollin in different ways. The possibility remains that some differences in the subsequent metabolism of 6a,7-dihydroxyphaseollin occur.

6a-Hydroxyphaseollin, the first product of phaseollin metabolism, was as fungitoxic as phaseollin to *C. lindemuthianum* race α2 (Van den Heuvel and Glazener, 1975), whilst the second metabolite, 6a,7-dihydroxyphaseollin, was as inhibitory as phaseollin to race δ11 only, but was only slightly inhibitory to the three other races. This compound was also toxic to *Cladosporium cucumerinum* (Bailey, 1974). Since the dihydroxyphaseollin is further metabolized in cultures of *C. lindemuthianum*, 6a-hydroxyphaseollin and 6a,7-dihydroxyphaseollin may be assumed to be intermediates in an overall detoxification process (Burden et al., 1974). The slow metabolism of the latter compound by race δ11 may be due to the relatively high sensitivity of this race to this metabolite.

It is unlikely that the differences in rate of conversion of phaseollin or in sensitivity to phaseollin or to its metabolites found in vitro between different races of *C. lindemuthianum* are large enough to explain the differential pathogenicity of these races to different bean varieties. Bailey (1974) has shown that phaseollin is the most prominent phytoalexin accumulating in bean hypocotyls infected with *C. lindemuthianum*. Hence no attempt is made to compare the metabolism of other phytoalexins by different races of this fungus.

If the mechanism of detoxification of phaseollin and other phytoalexins is operating in susceptible interactions between bean and *C. lindemuthianum*, this could explain the low levels of phytoalexins in spreading lesions found by Bailey (1974). Metabolic products of phaseollin may then be present in such tissues. In preliminary experiments (unpublished results), only once a small amount (equivalent to c. 1 μg/g fresh weight of tissue) of 6a-hydroxyphaseollin was detected in extracts of bean hypocotyls bearing spreading lesions of *C. lindemuthianum* race α2 seven days after inoculation. Diffusates of bean pod cavities infected with this race contained the two phaseollin metabolites. In comparable infection-droplets Cruickshank et al. (1974) detected a conversion product of phaseollin, designated as Ph 'X', which most likely was identical with 6a-hydroxyphaseollin, as compared by UV and mass spectral data. As bean pod diffusates may simulate in vitro rather than in vivo conditions, there is, as yet, only little evidence that the metabolic conversion of phaseollin by *C. lindemuthianum* is an important factor in the interaction between bean and this pathogen.

Samenvatting

Omzetting van phaseolline door verschillende fysio's van Colletotrichum lindemuthianum

Een vergelijkend onderzoek werd verricht naar het vermogen van vier fysiologische rassen van het bonepathogeen *Colletotrichum lindemuthianum* om het fytoalexine phaseolline om te zetten.

In schudculturen waaraan 10 μg phaseolline/ml was toegevoegd, werd dit door alle fysio's op gelijke wijze omgezet, hoewel met verschillende snelheid (Fig. 1). Steeds werd phaseolline eerst omgezet tot 6a-hydroxyphaseolline, en dit produkt vervolgens tot een verbinding die geïdentificeerd kon worden als 6a,7-dihydroxyphaseolline. Hierna konden geen verdere produkten worden aangetoond.

6a,7-Dihydroxyphaseolline was even fungitoxisch als phaseolline voor fysio δ11, maar was slechts weinig fungitoxisch voor de fysio's α1, α2 en γ1 (Tabel 1).

De verschillen in omzettingssnelheid van phaseolline en in gevoeligheid voor phaseolline of zijn omzettingsprodukten die tussen de fysio's gevonden zijn, zijn onvoldoende om de fysiospecifieke interacties tussen de boon en de verschillende fysio's van *C. lindemuthianum* te verklaren.

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