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DEGRADATION OF THE ISOFLAVONE BIOCHANIN A BY ISOLATES OF *NECTRIA HAEMATOCOCCA* (*FUSARIUM SOLANI*)

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Key Word Index—*Nectria haematococca*; *Fusarium solani*; isoflavonoids; biochanin A; pisatin; degradation; phytopathogenic fungi.

Abstract—Twelve isolates of *Nectria haematococca*, mating population VI (*Fusarium solani*) previously characterized for their virulence on pea plants and their ability to degrade the phytoalexin pisatin were assayed for the catabolism of the isoflavone biochanin A (5,7-dihydroxy-4′-methoxyisoflavone). Eleven isolates catabolized the isoflavone along the pathway: biochanin A → dihydrobiochanin A → 3-(*p*-methoxyphenyl)-6-hydroxy-γ-pyrone → *p*-methoxyphenylacetic acid → *p*-hydroxyphenylacetic acid → 3,4-dihydroxyphenylacetic acid.

INTRODUCTION

Higher plants possess a variety of defense mechanisms to prevent the penetration and the intracellular growth of fungal parasites. Among these mechanisms, the structurally diverse group of preinfectional inhibitors [1, 2], various other polyphenols [1] and the ever increasing number of phytoalexins [3–6] are considered to be important defense compounds.

Several phytopathogenic fungi have recently been shown to possess the ability to degrade the defense compounds of their hosts [7–9]. Though not all successful pathogens can be considered to possess the ability to degrade phytoalexins [9, 10], recent studies with numerous isolates of *Nectria haematococca*, mating population VI (*Fusarium solani*), have established an association between the ability to degrade the pea phytoalexin pisatin and virulence on peas [11, 12]. All highly virulent strains were tolerant of pisatin and were able to detoxify the phytoalexin by *O*-demethylation and degradation.

In a series of other studies, numerous fungi of the genus *Fusarium* were shown to be potent degraders of both preinfectional inhibitors and phytoalexins with an isoflavonoid skeleton [7, 13–16]. Thus, *Fusarium javanicum* converted the isoflavone biochanin A (5,7-dihydroxy-4′-methoxyisoflavone) (**1**) first to dihydrobiochanin A, which was subsequently degraded via 3-(*p*-methoxyphenyl)-6-hydroxy-γ-pyrone and *p*-hydroxyphenylacetic acid to 3,4-dihydroxyphenylacetic acid [15]. Other *Fusarium* species start biochanin A degradation by 4′-*O*-demethylation yielding genistein [17] or by 3′-hydroxylation [18].

In this study, we have determined the ability of 12 isolates of *N. haematococca* to degrade the isoflavone biochanin A (**1**). This compound is occasionally considered to be a preinfectional inhibitor [19–21] and it was shown to occur in considerable concentrations in the rhizodermis of a variety of plants (e.g. *Cicer arietinum*, various *Trifolium* species, *Baptisia australis* and *Ononis spinosa*) of the Leguminosae [22, 23]. The 12 selected isolates have previously been shown to differ in their ability to degrade pisation and in their virulence on pea [11]. In addition, one on the isolates (T-30) is insensitive to biochanin A and is pathogenic on chickpea *Cicer arietinum* [24], a plant that produces biochanin A. We wanted to determine whether these isolates of *N. haematococca* also differ in the ability to degrade the preinfectional inhibitor biochanin A. Furthermore, the degradative pathway of **1** by *N. haematococca* and the sensitivity of this fungus to **1** were elucidated.

RESULTS

In three parallel series of standard incubation assays with mycelial preparations of the 12 isolates of *N. haematococca* [11], the breakdown of biochanin A (**1**) (10^{-4} M) was followed for a period of up to 48 hr. Aliquots of the nutrient medium taken at 2 hr intervals were assayed for biochanin catabolites by TLC (S_1, S_2), UV-visible spectroscopy and spectroscopic means as described previously [15]. Thus, the chemical structures of the individual metabolites and the time period of their transient accumulation were determined. The structures of the degradation products are depicted in Fig. 1 where they are arranged in a sequence as indicated by the times of their maximum accumulation (Table 1). Except for isolate T-214 which seemed to lack the ability to degrade **1** all other isolates rapidly degraded the isoflavone. After a lag phase of some 2–3 hr, dihydrobiochanin A (**2**) was observed as the first conversion product with the pyrone, **3** being formed as the subsequent catabolite. Finally, *p*-hydroxyphenylacetic acid (**5**) and 3,4-dihydroxyphenylacetic acid (**6**) could be isolated from the nutrient medium. *p*-Methoxyphenylacetic acid (**4**) was detected in small amounts in three isolates only. Using [2-^{14}C]biochanin A as substrate for several isolates the acids **5** and **6** could be isolated in labelled form which proves that the acetic acid side chain of **5** and **6** originates from carbon atoms 2 and 3 of biochanin A. Though **6** was not accumulated by the isolates T-8, T-30, T-78 and T-110, we assume that in these cases *p*-hydroxyphenylacetic acid is also catabolized via 3,4-dihydroxyphenylacetic acid.

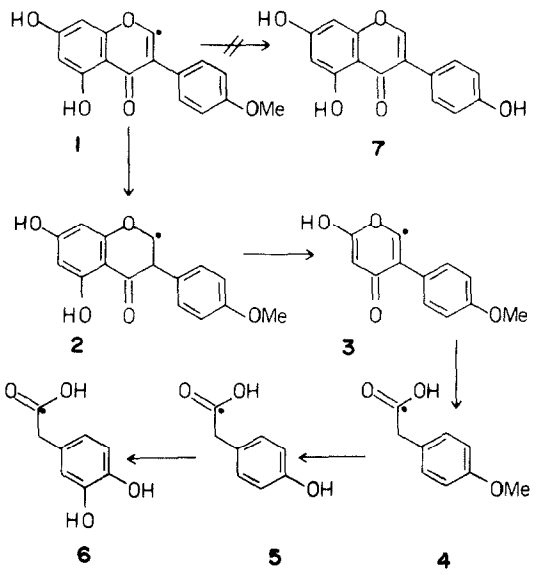

Fig. 1. Degradative pathway of biochanin A as carried out by isolates of *Nectria haematococca* in the sequence indicated by time periods of their maximum accumulation. Genistein (7) formation could not be detected with any isolate.

Genistein (7), which is the first degradation product of biochanin A with various other *Fusarium* species [13, 17, 25], could not be detected with any of the *N. haematococca* isolates used in these studies.

The mycelial growth rate of all 12 isolates was essentially unaffected (7 to −5% inhibition) by biochanin A (1×10^{-4} M).

DISCUSSION

Eleven out of the 12 isolates of *F. solani* investigated in our studies rapidly degraded the preinfectional inhibitor biochanin A. The degradation of 1 again appeared to be substrate induced. Although the 11 isolates differ greatly in their ability to degrade the phytoalexin pisatin (Table 1), there seems to be no significant qualitative or quantitative difference in the ability to degrade a 5-hydroxyisoflavone. These data suggest that the ability for phytoalexin degradation might be a more specific attribute than the ability to degrade a preinfectional inhibitor such as biochanin A. However, isolate T-214 apparently lacks the ability for the degradation of both biochanin A and pisatin. The observation that this isolate was just as tolerant of biochanin A as were isolates that could degrade this compound was somewhat surprising. This could mean that degradation of biochanin A is not important for tolerance to biochanin A, or possibly degradation of biochanin A did not occur under the cultural conditions used for the metabolism studies but did under the cultural conditions used to bioassay T-214 for biochanin A sensitivity. Differences in cultural conditions have previously been shown to influence isoflavonoid degradation [11, 26].

Although not all fungi of the genus *Fusarium* which have so far been investigated [7, 13, 17] possess the ability for isoflavone degradation, the great majority of the isolates of *N. haematococca* (Table 1) are characterized by this property. Furthermore, all the isolates used in this study that degraded biochanin A did so along the same pathway as shown in Fig. 1. This metabolic sequence has so far only been shown to occur with *Fusarium javanicum* [15], while other *Fusarium* species either *O*-demethylate 1 to genistein (7), which is subsequently cleaved along different routes [13, 25], carry out 3′-hydroxylation of 1 [18], *O*-methylate 1 in position 7 [18], or use other so far undescribed initial reactions of isoflavone catabolism [13]. In general, studies of this type offer a means of investigating the great metabolic diversity of this group of fungi. Furthermore, the results corroborate that pathogenic strains of *Fusarium* species

Table 1. Catabolites of biochanin A produced by isolates of *Nectria haematococca* and the time period (hr) when maximum accumulation of these catabolites occurred.

Isolate*	Virulence on pea*	Degradation of pisatin*	Catabolites of biochanin A				
			Dihydro-biochanin A (2)	Phenyl-pyrone (3)	*p*-Methoxy-phenylacetic acid (4)	*p*-Hydroxy-phenylacetic acid (5)	3,4-Dihydroxy-phenylacetic acid (6)
T-8	High	+	8–12	30	n.d.	34	n.d.
T-27	High	+	4–12	12–30	n.d.	34	34
T-30	High	+	12–20	24–30	n.d.	34	n.d.
T-63	High	+	2– 6	12–26	n.d.	34	36
T-69	Very low	−	4–24	24–30	n.d.	34	34
T-78	Low	+	8–12	24–30	n.d.	32	n.d.
T-86	Low	+	12–30	24–30	n.d.	34	34
T-110	Very low	−	6–12	24–30	34	34	n.d.
T-200	Low	+	12–24	24–30	n.d.	34	34
T-214	Very low	−	No degradation of biochanin A observed				
T-224	Low	+	2–12	24–30	34	34	34
T-231	Very low	−	2–12	24–30	24	34	34

The time values represent the average of three determinations.

*Isolate number and data on the virulence of these isolates on pea plants as well as their ability to degrade the phytoalexin pisatin are from ref. [11].

n.d., Catabolite not detected.

possess the ability to metabolize those phenolic constituents of their host's which are accumulated in the living plant tissue in very high concentrations [22, 23].

EXPERIMENTAL

Reagents. Biochanin A, [2-^{14}C]biochanin A and reference samples of Biochanin A catabolites were from previous studies [15].

Growth of fungi. Nectria haematococca isolates [11] were grown on Czapek–Dox medium and prepared for degradative studies according to earlier reports [15, 17].

Standard incubation assays. Mycelia were collected by filtration, washed × 3 with KPi buffer (0.05 M; pH 7.5) and 3 g was inoculated into 100 ml of the same buffer containing biochanin A (10^{-4} M). Substrate was predissolved in 0.5 ml 2-methoxyethanol prior to transfer into the buffer. These flasks were incubated at 30° and 160 rpm.

Detection of catabolites. Samples (6 ml) of the standard incubation assays were drawn every 2 hr and mycelium removed by filtration. After acidification (5% H_2SO_4; pH 2–3) catabolites were extracted with Et_2O. The organic layer was recovered, Et_2O removed under vacuum and the residues were taken for TLC analysis as a methanolic soln. TLC was conducted on Si gel F_{254} plates with suitable reference compounds in $CHCl_3$–MeOH (10:1) (S_1) and HOAc–$CHCl_3$ (3:7) (S_2).

Catabolites were detected under a short wavelength UV-light and with spray reagents (fast blue salt B and diazotized *para*-nitroaniline). The preparative isolation and the structural elucidations of catabolites by UV, MS and NMR techniques have previously been described [15]. R_fs of the compounds are as follows. In solvent S_1: **1**, 0.53; **2**, 0.56; **3**, 0.27; **4**, 0.40; **5**, 0.15; **6**, 0.02. In solvent S_2: **1**, 0.88; **2**, 0.91; **3**, 0.65; **4**, 0.96; **5**, 0.6, **6**, 0.03.

Measurements of antifungal activity. The radial growth bioassay as described previously [24] was used to record the sensitivity of the isolates to biochanin A (1×10^{-4} M). Growth measurements were made daily over a 7 day period.

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