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CUCURBITACINS—REPRESSORS OF INDUCTION OF LACCASE FORMATION

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Key Word Index—*Botrytis cinerea*; laccase; induction; *Ecballium elaterium*; cucurbitacins; cucumber; repression of induction; pectolytic enzymes.

Abstract—It is demonstrated that some members of the Cucurbitacea contain compounds which inhibit the induction of laccase formation by *Botrytis cinerea*. The general properties of the inhibitory substances correspond to those of cucurbitacins. Cucurbitacin I and D inhibit induction of laccase formation as do the extracts of plant tissue. In extracts of *Ecballium* the inhibiting activity was tentatively identified as being due to the presence of cucurbitacins. The possible role of the cucurbitacins in plant tissue in relation to infection is discussed.

INTRODUCTION

In a previous paper [1], we showed that extracts of cucumber fruit were able to repress induction of laccase formation by *Botrytis cinerea*, under inducing conditions. We wished to determine whether this was a characteristic of other members of the Cucurbitacea and to try and identify the active substance present in the fruit. The presence of antifungal compounds of the phytoalexins type has not been reported in cucumbers. It has been suggested that coniferyl alcohol, which apparently forms on infection and if locally applied is toxic to fungi, might have a phytoalexin-like role [2]. The entire family is characterized by the presence of cyclic triterpenoids (lanosterol derivatives), which are collectively termed cucurbitanes or cucurbitacins [3, 4]. The presence of cucurbitacin C has been reported frequently in cucumber fruit [5, 6] and variation in the amount present in different cultivars is one criteria for selection of the fruits, since the cucurbitacins are responsible for the bitter taste of some of the cultivars. The cucurbitacins are pharmacologically active [3], they have activity as kairomones and as deterrents of insect feeding [7, 8], they may be sequestered by *Diabrotica* sp. [9], they are known to act as antimitotic agents [10] and they inhibit somatic embryo formation in some plants [11]. The activity of this group of compounds against fungi has not apparently been studied to any extent, although it has been reported that cucurbitacin C and related compounds could inhibit the growth of *Phytophthora cactorium* at concentrations of 10 mg/l [12]. It seemed possible therefore that cucurbitacins are responsible for the repression of laccase formation by *Botrytis*. *Ecballium elaterium*, the squirting cucumber, is a useful source of cucurbitacins and the compounds are easily extracted from it [3, 4]. We therefore used this fruit as a source of active compounds. In the following we will report that repression of laccase formation is not restricted to cucumber fruit extracts and that cucurbitacin I and D are also active repressors.

RESULTS AND DISCUSSION

We tested the effect of expressed juice and the residues of fruits of *E. elaterium* on laccase formation by *Botrytis* under inducing conditions. As can be seen from the results in Table 1, extracts of *Ecballium* are extremely active in repressing laccase formation under conditions in which the enzyme is normally found. Inhibitory activity was present both in the expressed juice of the fruit and in the solid residue. The active compounds present in the solid residue could be extracted into chloroform. Re-addition of the chloroform extract, after evaporation to dryness, effectively repressed laccase formation, the degree of repression being proportional to the amount of inhibitory compound added. Extraction of the juice, by shaking with chloroform prior to addition to the culture medium removed all inhibitory activity from the aqueous phase. The inhibitory activity could be recovered from the chloroform extract (data not shown).

A related species, *Cucumis prophetarium*, also contains compounds which are very active in repressing laccase formation. Expressed juice (3 ml/30 ml medium) from fresh fruit reduced activity from 2.83 μl O_2/ml medium/min to 0.6 after 10 days of culture. Powder (2 g dry powder/30 ml medium) of dried fruit was equally active and reduced activity from 2.01 to 0.2 μl O_2/ml/min after 13 days of culture.

Examination of extracts of *Ecballium* and *C. prophetarium* by TLC using the procedures of Gmelin [13, 14], showed the presence of a number of compounds corresponding in their R_f values to cucurbitacins including cucurbitacin I, which was compared with an authentic sample of this compound. In all the cases examined, fungal growth was not repressed in any way by addition of extracts of *Ecballium*, *Cucumis* or by the addition of pure cucurbitacin I. In some cases marked stimulation was observed.

We tested the addition of the extracts of *Ecballium* directly to the incubation mixture used to assay laccase

Table 1. Effect of extracts of *E. elaterium* on laccase formation by *B. cinerea* after 7 days of culture

Addition to medium	Enzyme activity ($\mu l\ O_2$/ml medium/min)	Dry wt (mg/flask)
Control	2.8	340
5 ml expressed juice	0.38	280
5 g solid residue (fresh)	0.56	590
5 g solid residue (dried)	0.33	625
$CHCl_3$ extract of solid residue*		
equivalent to 10 ml extract	0.93	440
equivalent to 25 ml extract	0.16	465
equivalent to 50 ml extract	0.11	460
5 ml expressed juice, aqueous phase, after extraction with $CHCl_3$	2.8	336

*90 g solid residue were extracted overnight with 100 ml $CHCl_3$. The extract was reduced to dryness and the residues dissolved in hot malt solution.

Dry wt of the cultures determined after 14 days of growth.

Table 2. Effect of time of addition of cucurbitacin on laccase formation by *B. cinerea*

Treatment	Activity ($\mu l\ O_2$/ml medium/min)			Dry wt (mg)
	3 days	6 days	9 days	
Control	0.55	2.18	2.8	296
+ ethanol (0.5 ml)	0.48	1.83	2.3	222
+ Cucurbitacin D, 2×10^{-4} M	0.65	1.2	1.4	208
+ Cucurbitacin I, 2×10^{-4} M	0.50	1.30	1.45	215
+ Cucurbitacin I, on day three*		0.87	1.3	241
+ Cucurbitacin I, on day five*		1.09	1.3	364

*The mycelium was removed on the day stated, and resown on fresh medium, containing the inhibitor. Laccase production had started, the level being 0.42 on day three and 1.6 on day five. The laccase activity associated with the medium was discarded.

Dry wt determined after 9 days.

activity. No inhibition was observed at the concentrations which inhibited appearance of laccase in the culture medium, confirming the results obtained with cucumber extracts [1].

As it seemed likely that cucurbitacins were the active compounds causing repression of laccase activity, we tested the effect of addition of cucurbitacin I or D on laccase formation. The compounds were dissolved in ethanol and added to the medium prior to autoclaving (Table 2). Both compounds inhibited laccase formation by 30–40% at the concentration used. In these experiments we could not raise the inhibitor concentration because these compounds are only sparingly water soluble. We also tested whether addition of cucurbitacin I was effective when laccase production by the fungus had started. Once production had begun, cucurbitacin was no longer effective in repressing formation of laccase (Table 2). We take this to indicate that cucurbitacin in some way interferes in the induction of laccase formation, i.e. affects the switching on of the relevant gene and not on the synthesis of the enzyme.

A chloroform extract of expressed juice of *E. elaterium* was prepared, evaporated to dryness, and the residue taken up in ethanol. Extract equivalent to 2.5 ml juice was chromatographed on TLC plates. Three zones were scraped off the plates, and extracted with ethanol (2.0 ml EtOH/zone): zone I (R_f 0.68), which corresponded with cucurbitacin I, and zones II and III (R_f 0.5 and 0.13), in which no reaction for cucurbitacins was detected. The extracts were reduced in volume to 0.5 ml and this amount applied per flask to cultures of *Botrytis* growing under inducing conditions and extracellular laccase determined after 10 or 14 days of growth. Only the results after 10 days are given, those after 14 days showing the same response to the different fractions. Addition of extracts from zones II and III resulted in laccase activities of 3.2 and 2.94 $\mu l\ O_2$/ml/min, cf. the controls 3.05 $\mu l/O_2$/ml/min or 2.94 after addition of ethanol activities equivalent to that of the controls. In contrast, addition of the extract from zone I resulted in reduced laccase activity, i.e. 2.28 $\mu l\ O_2$/ml/min, or 22% inhibition of laccase formation. This is the degree of inhibition expected from the amount of juice from *Ecballium*. The equivalent of 20 ml chloroform extract when added under the same conditions inhibited the formation of laccase by 71% (cf. also with the results in Table 1). We take this to

indicate that the inhibition of the formation of laccase activity by the juice of *Ecballium* is indeed due to the cucurbitacin present in this juice and not due to other compounds. The concentration of cucurbitacin I + D in *Ecballium* juice has been reported to be 0.3–0.5% [15]. Therefore the extracts from the fruits and the expressed juice would contain amounts of these compounds to give final concentrations in the culture medium of 2×10^{-4} M.

We also prepared a chloroform extract of cucumber fruit, by extraction overnight at room temperature. The extract of 220 g fresh weight of fruit was reduced to dryness and then taken up in ethanol. The ethanol extract was applied to cultures of *Botrytis* growing under conditions of induction of laccase formation. The appearance of laccase activity was reduced from 2.94 to 0.22 μl O_2/ml/min after 10 days of culture. The inhibitory activity of cucumber extracts, which we reported previously [1], can therefore be ascribed to substances which behave in a way similar to cucurbitacins. Unfortunately, we have not been able to obtain an authentic sample of cucurbitacin C, the compound reported to be present in cucumber fruit in varying amounts [5, 6], depending on the cultivar.

It was possible that the inhibition of laccase formation was due to some indirect effect, such as inhibition of breakdown of the second inducer of laccase formation, pectin [16]. We therefore assayed the effect of cucurbitacin I directly on the activity of pectinlyase and of polygalacturonase. The concentrations of the compound which inhibited laccase formation did not inhibit the activity of either enzyme as determined viscometrically.

Not all cultivars of cucumber are infected with equal ease by *B. cinerea*, and they also differ in their cucurbitacin content. It might therefore be speculated that the cucurbitacins are involved in some way in resistance to *Botrytis*, although we have only indirect evidence for this. This appears to be the first account of the effect of a cucurbitacin on the processes which lead to the formation of a specific enzyme by a fungus, without affecting the growth of the organism. This finding might have future applications in developing ways of controlling *Botrytis* infections, if indeed it can be established that laccase formation and virulence are causally related, as suggested by us previously [1].

EXPERIMENTAL

Botrytis cinerea was cultured as previously described [16–18], but in 100 ml Erlenmeyer flasks containing 30 ml medium. Medium for inducing laccase formation contained both gallic acid and pectin in addition to malt [16]. To the flasks suitable amounts of solid material or expressed juice were added. In all cases either the flasks were autoclaved after addition of the extracts, or in experiments where juice was added after inoculation the juice was boiled before addition. In the case of $CHCl_3$ extracts of the fruit, the extracts were prepared, a suitable amount evapd to dryness in the culture flasks and the medium then added. Cucumbers were purchased at a local supermarket and fruits of *Ecballium* collected from plants in the gardens of the Hebrew University. Cucurbitacin I was dissolved in $CHCl_3$ and then added to the flasks, the solvent evapd and the culture medium added and then autoclaved. Laccase activity was assayed using a Clark type oxygen electrode as previously described, using hydroquinol as substrate. The activity of pectolytic enzymes was determined by following the rate of flow of a soln of Na pectate through an Oswald viscometer, at 26°.

TLC was carried out as described in refs [13, 14]. Extracts were spotted onto silica gel plates, which were developed with $CHCl_3$–EtOH (9:1). The $CHCl_3$ contained 0.75% EtOH. The plates were dried, sprayed with $FeCl_3$, viewed in daylight and UV, then sprayed with 2% dimethylaminobenzaldehyde in 2 vol 84% phosphoric acid and 8 vol EtOH. They were then heated to 120° for 10–20 min and again viewed both in daylight and in UV. The R_f of cucurbitacin I was 0.68. At the end of the experimental period, the mycelial mat was removed, rinsed and its dry wt determined by drying at 105° to constant weight.

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