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EFFECT OF CUCURBITACINS ON mRNA CODING FOR LACCASE IN *BOTRYTIS CINEREA*

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Abstract—The effect of cucurbitacin and of *Ecballium* extract on the formation of mRNA coding for laccase was examined in cultures of *Botrytis cinerea* grown with inducers of laccase formation, in the presence or absence of the inhibitory compounds. RNA was isolated from the cultures and probed with specific DNA probes for laccase. As an internal control, the RNA was probed for *Botrytis* β-tubulin mRNA. From an analysis of the results it is clear that cucurbitacin I and *Ecballium* extract specifically repress the amount of mRNA coding for laccase. This could account for the previously observed repression of laccase formation by cucurbitacins.

INTRODUCTION

We have previously shown [1] that in the presence of cucurbitacins the amount of laccase protein produced by *Botrytis cinerea* is significantly reduced. We have also argued in a number of publications [2–5] that laccase produced by *B. cinerea* is part of the 'attack' arsenal used by the pathogen while invading its host. The activity of laccase in the culture medium of *Botrytis* is reduced concurrently with the amount of enzyme protein. This repression of the enzyme protein could be due to a direct effect on the level of mRNA which codes for laccase. We, therefore, have attempted to investigate this question, by extracting RNA from mycelium of *Botrytis* grown either under conditions leading to increased induction of enzyme activity or under non-inducing conditions in the presence or absence of either cucurbitacin or *Ecballium* extracts.

RESULTS AND DISCUSSION

Since isolation, investigation and quantification of RNA is quite difficult in fungal mycelium, the procedures finally adopted are described in some detail in the Experimental section. Among the difficulties encountered were the presence of polysaccharides secreted by the fungus and the formation of quinones derived from the inducer, gallic acid, which becomes oxidized during growth of mycelium.

To compare the amount of the mRNA coding for laccase with an internal standard we used β-tubulin mRNA. Since the fungus grows normally in the presence of amounts of cucurbitacin which repress laccase formation, the amount of mRNA coding for tubulin should not vary appreciably due to the treatment with cucurbitacin. In the first experiment we used RNA dot blots of total RNA, challenging the blots with either the DNA probe for laccase or with a DNA probe prepared against yeast tubulin. The results are shown in Fig. 1.

The data in Fig. 1 clearly indicate that the amount of mRNA coding for laccase is markedly repressed by culture of the fungus in the presence of cucurbitacin, which in these experiments was the highly active triacetate of cucurbitacin D [3]. We also looked at the

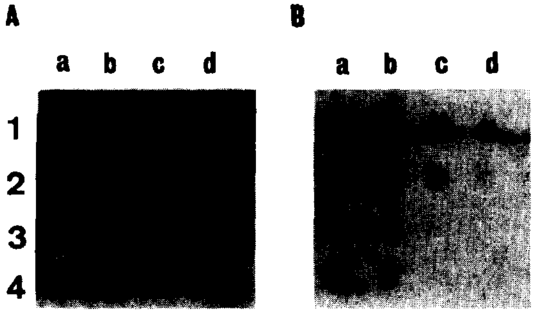

Fig. 1. RNA dot blots onto nitrocellulose of serially diluted total RNA preparation. (A) Hybridization with yeast tubulin probe. (B) Hybridization with *Botrytis* laccase probe. Dilutions are 4-fold a–d. 1. mRNA from induced medium. 2. Inducing medium + 2,16,25-cucurbitacin D triacetate (0.16 mM). 3. Non-inducing medium (malt). 4. Non-inducing medium with cucurbitacin as in 3.

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Table 1. Effect of gallic acid and pectin as inducers of laccase mRNA expression in *Botrytis cinerea* (the X-ray films were examined by quantitative scanning densitometry)

Experiment number	Tubulin mRNA induced/non-induced	Laccase mRNA induced/non-induced
1.	0.82	>50
2.	1.27	>50
3.	1.27	24
4.	n.c	1.86
5.	n.c	2.18

n.c. not checked.

effect of the inducers on the expression of laccase and tubulin mRNA, by analysing the results of a number of dot blots (Table 1). Expression of laccase mRNA in cultures grown in the presence of inducers was substantially higher than in cultures grown under non-inducing conditions. No significant differences were observed with tubulin mRNA. Some variability in the results on the effect of the inducers of laccase activity on the level of mRNA is seen in Table 1, which can probably be ascribed to the differences in the age of the mycelium used in the different experiments (4 days in experiments 1–3 and 5 days in experiments 4 and 5). Since laccase formation is strongly time-dependent it is to be expected that the level of mRNA coding for it will also be so dependent. Furthermore, it has previously been shown [5] that *Botrytis* contains at least two distinct laccases and great variability of laccases in the fungus is also indicated by a recent report by Slomczynski *et al.* [6]. In this respect the results on *Cryphonectria* are also relevant [7]. It was shown that when a specific laccase gene was deleted, a previously undetected inducible extracellular laccase appeared. These observations could be related to the fact that residual mRNA coding for laccase was found in the dot blots following cucurbitacin treatment (Fig. 1). It is possible that different laccase genes are repressed differentially by cucurbitacins.

Dot blots are not totally reliable and can easily lead to artefacts. Despite the encouraging results shown in Fig. 1, we decided to carry out further experiments, but with marked differences. In addition, we now used a probe against β-tubulin from *Botrytis* itself.

The laccase activity of a number of cultures used in the next experiment are shown in Table 2. The mycelia were harvested in this case after eight days and total RNA was extracted. The RNA was separated by gel electrophoresis.

Both cucurbitacin E and I were very poorly soluble in the medium and hence quite large amounts were used. In previous work we found that the crude *Ecballium* extract was a more suitable inhibitor [8].

Northern blots of one of these separations were now carried out using the DNA probes. The results of the hybridization are shown in Fig. 2. Figure 2a shows the hybridization using the laccase probe and Fig. 2b shows that using the tubulin probe. In each case lanes 1–3 show the mRNA from mycelia grown in the presence of cucurbitacins, while lane 4 shows the mRNA from the induced culture, grown in the absence of cucurbitacin. The autoradiograms of the Northern blots were analysed by quantitative scanning densitometry in order to evaluate the effect of treatment of cucurbitacin on the levels of the mRNAs. This analysis showed that the ratio of intensity of labelling with the laccase probe as compared with the tubulin probe was 1.32 in the control cultures, while it was 1.02 in the cultures treated with cucurbitacin I and 0.31 in those grown in

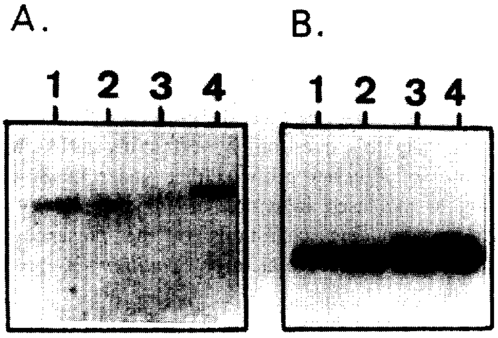

Fig. 2. Northern blots of RNA extracts as described in Fig. 2. (A) Hybridization using laccase probe. (B) Hybridization using *Botrytis* β-tubulin probe.

Table 2. Inhibition of extracellular *Botrytis* laccase formation by cucurbitacins. The mycelium from these cultures was used for subsequent RNA extraction. Laccase activity as μl O_2 min^{-1} ml $medium^{-1}$

	Laccase activity Days of culture		% Inhibition Days of culture	
Culture conditions	7	8	7	8
1. Control (no addition)	3.2	3.8	0	0
2. + Cucurbitacin E 45 mg/150 ml medium	0.55	1.64	83	57
3. + Cucurbitacin I 45 mg/150 ml medium	0.65	1.64	80	57
4. + *Ecballium* juice 10 ml/150 ml medium	0.55	1.36	83	64

the presence of the *Ecballium* extract. No clear cut effect was observed with cucurbitacin E. The reason is not entirely clear but may be related to the time course of the inhibition. It should be noted that the maximal inhibition was observed on day 7, but the mycelium was harvested on day 8 when laccase activity began to rise (Table 2). The fact that the *Ecballium* extract was much more active than pure cucurbitacin I may suggest that the mixture of cucurbitacin is more effective than a single compound. The possibility that the laccase probe is active towards a single laccase, while the mycelium can form several different laccases cannot be ignored [5, 6]. Nevertheless, the results are consistent with those observed in the dot blots. The effect of cucurbitacins on the amount and activity of laccase in *Botrytis* can be ascribed to their effects on the level of mRNA.

It must be remembered that since the amount of laccase secreted, and present in the mycelium, changes with time of culture, the precise moment at which the samples are taken is crucial. It is extremely likely that if a precise time curve of mRNA levels coding for laccase were prepared we would observe periods at which the repression would be very high, and at other periods when the synthesis of the enzyme is low when repression would be minimal. We still do not know by which mechanism the mRNA level is repressed. It could be due to an inhibition of transcription, perhaps due to specific binding of the inhibitor to certain regions of DNA. However, at this stage we cannot rigorously exclude the possibility that the reduced level of the mRNA is due to a specific effect on the stability of the mRNA coding for laccase, e.g. due to its more rapid turnover and/or degradation. The isolation procedures for the mRNA were extremely laborious, so that we were unable to verify these latter points in the course of this work. It is interesting to note that a recent report on cytotoxicity of cucurbitacins towards various human tissues shows differences in the sensitivity of tissues to same amount of active compound [9]. This at least is slightly reminiscent of our results of a differential effect on different enzymes in the same tissue. Although there have been a number of recent discussions of laccases, particularly from the point of view of their bioinorganic chemistry [10, 11], these have not added anything to our understanding of their biological function. Perhaps more relevant is the paper showing induction of PPO by systemin [12], and a similar mechanism might operate for induction of laccase in fungi, due to host signals.

EXPERIMENTAL

Culture of Botrytis *and assay of laccase activity* was as previously described [1–4, 8], on malt only or with additions. All the cucurbitacins were added to the culture medium, dissolved in 0.4 ml MeOH at the time of inoculation [2] except for 2-*O*-β-D-glucopyranosyl-cucurbitacin I, which was dissolved in 3 ml of H_2O.

Ecballium *extract* was prepared by expressing the juice of freshly harvested *Ecballium* fruit, using a juice separator, freeze-drying the juice and redissolving the extract in H_2O. Other cucurbitacin prepns were prepd as previously described [1–4].

RNA isolation from Botrytis cinerea. RNA was extracted according to the methods used in Dr J. Van Kan's laboratory in the Department of Phytopathology, Wageningen. Mycelium was removed from the medium, washed several times with sterile H_2O, dried on filter paper and frozen in liquid N_2.

Frozen mycelium was ground to a powder in liquid N_2 with pestle and mortar. The powder was transferred to a sterile 50 ml plastic tube and to 1 g mycelium 2 ml guanidine buffer were added (8 M guanidine hydrochloride; 20 mM MES, 20 mM EDTA, 50 mM mercaptoethanol, pH 7). This was mixed with a Vortex stirrer for 2 min.

An equal vol. of phenol–$CHCl_3$–isoamyl alcohol (25:24:1) was added and the soln again vortexed for 2 min.

The mixture was transferred to a sterile centrifuge tube and centrifuged for 45 min at 10 000 rpm using a Sorvall SS-34 rotor at 4°. The aq. upper phase was transferred to a sterile 50 ml tube and 1 vol. of $CHCl_3$–isoamylalcohol (24:1) was added. The mixture was vortexed for 2 min and centrifuged for 15 min as before. The aq. upper phase was transferred to 15 ml Corex tubes and 1/3 vol. of 8 M LiCl was added. Samples were kept at 4° overnight. The next day the samples were centrifuged for 30 min at 11 000 rpm using a Sorvall HB-4 rotor, at 4°. The pellet was washed once with cold 2 M LiCl and twice with cold 70% EtOH and centrifuged after each wash for 15 min at 11 000 rpm as before. After the last wash, the RNA pellet was dried in air and dissolved in H_2O containing 0.1% diethyl pyrocarbonate (DPC). The amount and purity of RNA was estimated from the *A* at 260 nm and the ratio of *A* 260 nm/280 nm.

Separation of RNA on agarose gels. Suitable samples containing 20–25 μg of total RNA for each lane were taken from each lot of RNA. RNA was precipitated overnight at −20° with 1/10 vol. of 3 M NaOAc pH 5.2 and 3 vol. of EtOH. The next day the RNA samples were centrifuged in an Eppendorf centrifuge at maximum speed for 30 min at 4°. The supernatant was discarded and the RNA pellet, of 20–25 μl, dissolved in 15–20 μl of freshly prepared RNA sample-buffer (200 μl de-ionized formamide, 70 μl HCHO, 40 μl 10*MOPS buffer, 40 μl dye (80% glycerol, 0.2% bromophenol blue; 1 μl ethidium bromide soln, (from a stock of 10 mg/ml). RNA samples were denatured by heating at 65° for 10–15 min, and then placed on ice till applying to the gel.

Northern blots. Gels for Northern blots were prepared as follows: For a 60 ml mini-gel Hoeffer apparatus, 1.2% agarose gel was made as follows: 0.72 g agarose were dissolved in 48 ml DPC, 0.1%, by heating to boiling. The soln was cooled to 40–50° and then 6 ml 10*MOPS buffer (0.4 M morpholino propane sulfonic acid, pH 7, 0.1 M Na acetate, 10 mM EDTA pH 8) and 6 ml formaldehyde were added. The gel was poured and left to solidify for about 20 min. The gel was run with 1 * MOPS as running buffer at 70–80 V for 2–3 hr. Electrophoresis was stopped when the tracking dye reached the bottom of the gel.

Blotting and hybridization. The gels were viewed under UV light and then soaked for 30 min in sterile H_2O, followed by soaking for 30 min in 10 * SSC. The transfer to nylon supported nitrocellulose membrane (Nitropure, MSI) was done overnight using 20 * SSC (3 M NaCl, 0.3 M Na citrate pH 7). The membrane was then briefly washed with 2 * SSC and baked for 2 hr in vacuum oven at 80°.

RNA-gels and Northern blots were carried out by the methods used in Dr R. Last's laboratory at Boyce Thompson Institute, and are modifications of standard procedures described in ref. [13]. Dot plot analysis was as described in ref. [14].

Plasmids. The two plasmids containing the inserts which were used us templates for the synthesis of radioactive probes for the hybridization reactions with *Botrytis* RNA were obtained from the laboratory of Dr J. van Kan, Wageningen (Van der Vlugt-Bergmans & J. van der Kan, unpublished results). The plasmid containing the entire open reading frame of *B. cinerea* β-tubulin gene and from Dr R. C. Stables, Boyce Thompson Institute, Ithaca for the laccase gene.

The plasmid containing the tubulin gene from *Botrytis* was introduced into *E. coli* DH5α by electroporation according to the protocol of Biorad for the Bacterial Transformation, Biorad Instruments.

The tubulin gene insert was cloned in Bluescript plasmid, pTUB56. The plasmid was cut with restriction enzymes PstI and BamHI (37°, 2 hr). A fragment of 1.7 kb from this cutting, which includes 75% of the tubulin coding sequence, was isolated from 1% agarose TAE (0.04 M Tris–acetate and 1 mM EDTA) gel, cleaned using the Geneclean kit (Bio 101 Inc.), and used as a template for radioactive-probe synthesis.

The plasmid containing the laccase gene consisted of a 1.3 kb insert (isolated from c-DNA library, GenBank Accession Number U20192)) of *B. cinerea* and was cloned in Bluescript, pBLccD48. The plasmid was cut with restriction enzymes ClaI and XhoI (37°, 2 hr) and the 1.3 kb insert cut from 1% agarose TAE gel, cleaned with the Geneclean kit, and used as a template in the synthesis of radioactive probe.

Radioactive probes. The probes were prepared using the Amersham Megaprime DNA labelling system (RPN 1606) and labelled with [^{32}P]-d-CTP obtained from Rotem Industries, Israel. Hybridization was carried out according to ref. [15], with slight modifications. Prehydridization was done in 0.1 ml/cm^2 Church buffer (7% SDS, 1% BSA, 1 mM EDTA, 250 mM NaPi buffer, pH 7.2) for 1–2 hr at 57° in a rotating hybridization oven, (Hybaid). Denatured radioactive probes were added to the hybridization bottles (without changing buffer) and incubated at 57° for 12–24 hr in the rotating oven, usually overnight. Washes were done as follows: 3× in 100 ml 6 * SSC, 0.1% SDS, at room temp., for 10–15 min each, followed by two 100 ml washes in 0.5 * SSC, 0.1% SDS, at 60°, for 20–30 min each. For the laccase probe, two additional washes with 100 ml 0.5 * SSC, 0.1% SDS, at 60°, 20–30 min each, were done. The tubulin probe was washed twice with 100 ml 0.1 * SSC, 0.1% SDS, at 60–65°, for 20–30 min each. The filters were removed from the bottles, air-dried and wrapped with Saran-wrap. The membranes were exposed to X-ray film (AGFA-Curix RP-2) for various periods and the labelled bands analysed with a Zeineh soft laser scanning Densitometer (model SL-TRFF). Alternatively the membranes were exposed for short periods to screens which were developed and processed in the Phospho-imager (FUJIX; Bio-imaging analyser, BAS1000, MacBAS).

Maintenance of E. coli *containing the plasmids* was according to standard procedures [12].

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