Phytochemistry, Vol. 27, No. 8, pp. 2505–2509, 1988.
Printed in Great Britain.

0031-9422/88 $3.00 + 0.00

REPRESSION OF LACCASE FORMATION IN *BOTRYTIS CINEREA* AND ITS POSSIBLE RELATION TO PHYTOPATHOGENICITY*

NURIT BAR NUN, AVIVIT TAL LEV, EITAN HAREL and ALFRED M. MAYER

Department of Botany, The Hebrew University of Jerusalem, Jerusalem 91904, Israel

(*Received* 4 *January* 1988)

Key Word Index—*Botrytis cinerea*; laccase; enzyme induction; enzyme repression; pectin; EDTA; gallic acid; cucumber; pythopathogenicity.

Abstract—It is shown that addition of EDTA to the culture medium of *Botrytis cinerea* can repress laccase formation and that this effect if reversed by addition of copper. EGTA also repressed laccase formation, by a different mechanism. Pretreatment of cucumber fruit with EDTA reduced the infection by *Botrytis* and prevented laccase formation. Cucumber fruit contained substances which were able to repress laccase formation. Resistance to *Botrytis* and the ability of extracts of the fruit to repress laccase formation were correlated, as were low levels of laccase in strains with fungicide resistance and low infectivity. The observations suggest that laccase formation is a necessary but insufficient requirement for infection by *Botrytis*.

INTRODUCTION

In a number of papers we have described the induction of laccase formation by *Botrytis cinerea* in culture and the properties of the enzyme [1–5]. Induction of laccase formation seems to be a more general phenomenon among many fungi [6, 7]. We have suggested that in the case of *Botrytis*, laccase formation may be closely linked with phytopathogenicity of the fungus since infection and laccase secretion appear to be correlated [5, 7]. The ability to form different laccases may play a part in the ability of *Botrytis* to infect a very wide range of hosts. In other fungi too, laccase formation and infectivity may be connected [8]. Other functions for laccase which have been suggested are a possible role in fungal morphogenesis [9], involvement in delignification and in spore formation [7].

It seemed therefore to be important to determine whether enzyme formation can be repressed and whether such repression affects the ability of *Botrytis* to infect its host. In the following we report on studies on repression of laccase formation in *Botrytis*.

RESULTS

There are very few specific inhibitors of laccase activity. Many inhibitors are copper chelators, among them EDTA which has been reported to inhibit laccase activity [10] and is not toxic to *Botrytis*. We therefore decided to test its effect on laccase formation. EDTA was added to the culture medium of *Botrytis* and its effect on enzyme formation and fungal growth followed. At the same time we compared its effect with that of EGTA a weaker chelator of copper, but an effective chelator of calcium, which is often a regulating ion in cellular processes.

The earlier studies on the inhibition of partly purified laccase induced by grape juice, from *Botrytis* showed some inhibition by 3 mM EDTA. However the laccase induced by gallic acid + pectin in these experiments was not inhibited even by the addition of 24 mM EDTA to the reaction mixture. The difference is probably due to differences between the two laccases [3, 4], such as the inducers used to cause their formation and consequently their molecular properties.

The results on the inhibition of laccase formation are shown in Tables 1–3. These results were obtained on basic medium and malt with (Tables 1 and 2) and without the addition of gallic acid (Table 3). It can be seen that EDTA almost totally represses laccase activity in the growth medium, while EGTA was far less effective. The effect of EGTA was partly reversed by calcium, while that of EDTA was totally reversed by Cu^{2+}, (Table 3) but not by Ca^{2+}. The inhibition was not due to some complex formation between the enzyme and EDTA and could not be reversed by prolonged dialysis. Laccase was not only absent from the culture medium, its level in the fungal mycelium was also repressed. *Botrytis* grown in the presence of EDTA at a concentration which repressed appearance of laccase in the culture medium contained only 10% of the laccase present in the mycelium of the controls. It is extremely likely that EDTA reduces availability of Cu^{2+} in the culture medium and therefore prevents formation of active enzyme. Indeed, availability of copper might be limiting laccase formation, since addition of copper in the absence of EDTA slightly increased its level in the medium. It should be noted that neither EDTA nor EGTA affected fungal growth in any way when both inducers were present in the culture medium (Tables 1–3), but some inhibition of growth by 12 mM EDTA was observed when only gallic acid was present (Table 1). It is also important to note that copper

*Dedicated to the memory of Tony Swain, whom we will miss as a distinguished contributor to the field of phytochemistry, both as scientist and as editor of *Phytochemistry*, and in appreciation of a long-standing friendship.

Table 1. Effect of EDTA on laccase formation and growth of *Botrytis cinerea*

Addition to medium	Enzyme activity μl O_2/ml/min	Growth g dry wt/culture
Pectin, 1.6%	2.73	1.54
EDTA, 12 mM	0	0.4
Pectin + EDTA 12 mM	0	1.25
Ca^{2+}, 36 mM	0.16	1.04
Ca^{2+},48 mM	0.24	1.03
Pectin + Ca^{2+}, 36 mM	2.76	1.53
Pectin + Ca^{2+}, 48 mM	2.91	1.61
Pectin + EDTA, 12 mM + Ca^{2+}, 36 mM	0.05	1.34
Pectin + EDTA, 12 mM + Ca^{2+}, 48 mM	0.05	1.15

The culture medium contained 2% malt and 0.1% gallic acid in all cases as well as buffer.pH was adjusted before inoculation if necessary, after addition of EDTA or copper. Enzyme activity was measured as described in experimental, after 12 days growth of culture. In the presence of malt and gallic acid the total activity was 0.4 μl O_2 /ml/min and the fungal dry weight after 12 days 0.8–0.9 g/culture.

Table 2. Effect of EGTA on laccase formation and growth of *Botrytis cinerea* and its reversal by Ca^{2+}

Addition to medium	Enzyme activity μl O_2/ml/min	Growth g dry wt/culture
Pectin	2.4	1.47
EGTA, 12 mM	0	1.14
Pectin + 12 mM EGTA	1.3	1.70
Ca^{2+}, 36 mM	0.11	1.07
Ca^{2+}, 48 mM	0.27	0.92
Pectin + Ca^{2+}, 36 mM	2.94	1.70
Pectin + Ca^{2+}, 48 mM	2.80	1.61
Pectin + EGTA + Ca^{2+}, 36 mM	2.61	1.65
Pectin + EGTA + Ca^{2+}, 48 mM	3.05	1.66

Culture conditions and enzyme activity as in Table 1. Culture medium contained malt 2% and gallic acid, 0.1%. Activity measured after growth of culture for 12 days.

Table 3. Effect of Cu^{++} on the repression by EDTA of laccase formation by *Botrytis cinerea*

Addition to medium	Enzyme activity μl O_2/ml/min	Growth g dry wt/culture
Cu^{2+}, 5 mM	0	0
Cu^{2+}, 10 mM	0	0
Gallic acid + Cu^{2+}, 5 mM	0	0
Gallic acid + Cu^{2+}, 10 mM	0	0
Pectin + Cu^{2+}, 5 mM	0.59	1.22
Pectin + gallic acid + Cu^{2+}, 5 mM	6.4	1.39
Pectin + gallic acid	3.05	1.21
Pectin + gallic acid + EDTA, 6 mM	0	1.12
Pectin + gallic acid + EDTA, 6 mM + Cu^{2+}, 5 mM	5.4	1.22

Basic medium only contained malt. Pectin, gallic acid EDTA or Cu^{2+} were added to the medium. Enzyme activity was measured after 14 days of growth of culture. Cu^{2+} was added as the chloride.

was highly toxic in the absence of EDTA and of pectin. When pectin was present, copper toxicity was not observed, probably due to binding of copper by pectin [11]. Apparently the copper requirement for growth is very low and the complex of pectin with small amounts of copper in the normal medium can furnish copper for laccase formation. However the binding of the limited amount of copper in normal medium, by EDTA is so tight that the copper is not available for laccase. The effect of EGTA is probably by a different mechanism than that of EDTA.

The results described above showing that it is possible to repress laccase formation without inhibiting fungal growth led us to conduct some experiments in which we tried to inhibit laccase formation by *Botrytis* during host infection. We therefore attempted to treat host tissue with EDTA prior to infection. The test tissue were in the first instance cucumber fruit as previously described [5]. A window of $1.0 \times 0.5 \times 0.4$ cm was cut into intact fruit after surface sterilization with 2% hypochlorite. Into the well either a 12 mM solution of EDTA, pH 6.0 was placed for 24 hr or in the controls, distilled water. At the end of this period the wells were infected with a suspension of fungal mycelium. Spread of the fungus into the fruit and laccase activity in the infected tissue were then followed. In the normally infected tissue an activity of 0.97 μl O_2/mg protein/min could be measured after 14 days of infection at the edge of the well. In this fruit the fungus could be seen to have spread throughout the tissue. In the fruit pre-treated with EDTA enzyme activity was barely detectable, 0.01 μl O_2/mg protein/min and virtually no hyphae were observed. This result was observed in numerous replications of the experiment. The absence of invasion of the host in the EDTA-treated fruit was borne out by electron microscopic observation of the fruits (results not shown here). In tissue into which the fungal mycelium penetrated typical dark colouration at the junction of cell walls was observed, prior to the onset of tissue disintegration. This discolouration was totally absent in EDTA-treated tissue. In the former mycelium could be readily observed and at the later stages fungal sporulation was observed. Both these were absent in the EDTA-treated tissue. Attempts to treat seedlings of pea, bean or melon by spraying them with EDTA prior to infection gave less clear cut results, but in all the cases the EDTA treatment significantly delayed, by three to five days, abscission of the cotyledons and leaves following infection. However infection in this case was not prevented.

In previous experiments we had observed that extracts of cucumbers, could in some cases induce laccase formation in the absence of other inducers [5]. When these experiments were repeated considerable variability was observed and in some cases no induction could be obtained. There appeared to be a correlation between the ability of cucumber extracts to induce laccase formation and their susceptibility to infection by *Botrytis*. The more susceptible the fruit were the better their extracts induced laccase formation. When, following infection, spread and penetration of the fungus into the host were delayed, the extracts were also poor inducers of laccase formation. These observations suggested that some cucumber varieties might contain inhibitors of laccase or compounds which repress its formation. We therefore added extracts from cucumber to the culture medium in the presence of the standard inducers, gallic acid and pectin. Two fractions from the cucumber were tested. The cucumbers were ground in a juice extractor and the juice or the solid residues added to the culture medium. The results of a typical experiment, repeated dozens of times, are shown in Table 4. The data clearly show that the cucumber preparation contained substances which either inhibited laccase formation or its activity. Inhibition was observed throughout the culture period and the time course of laccase formation, at the reduced level, was the same as that of the controls. Maximal activity was always obtained after *ca* 12–14 days of culture. Inhibition was not due to an inhibition of the growth of the fungus. The data in Table 4 clearly show that the cucumber extracts promoted fungal growth as determined by the dry weight of the mycelial mass. The inhibition of laccase was due to repression of its formation and not due to inhibition of laccase activity itself, after it had been secreted into the medium. Preparations of cucumber extracts were added directly to an active enzyme preparation, in the assay system and activity measured. Even when the assay system contained 50% crude cucumber extract or 30% cucumber juice, no inhibition was observed.

The degree of inhibition of laccase formation by the extracts increased with the amount added. Thus 18.5% solid residues added to the medium, resulted in laccase activity of 0.93 μl O_2/ml medium/min, 33% solid residues reduced this to 0.65 μl O_2/min, and 48% gave a residual activity of 0.4 μl O_2/min in the culture medium. The activity of the controls, when only inducers were present was about 6.0 μl O_2/min/ml medium. The curve of acitivity against concentration of solid residues was asymptotic.

Table 4. Effect of cucumber juice and cucumber solid residues on laccase formation by *Botrytis cinerea* in the presence of pectin and gallic acid

Growth medium	Enzyme activity	Growth
	μl O_2/g dry wt	g dry wt/culture
Malt + 2 inducers	3.3	1.84
Malt + 2 inducers +'solid residue'	0.16	2.68
Malt + 2 inducers + 'juice'	0.35	3.1
Malt 2 inducers + 'solid residue' +juice	0.09	3.4

Solid residue was added 350 g/l medium and juice 900 ml/l medium. Activity measured in medium of cultures after 14 days of growth.

It seemed possible that the cucumber preparations acted on the step at which laccase is secreted from the mycelium and not on its formation. This possibility could be excluded by direct measurements of intracellular enzyme activity in the mycelium (Table 5). Clearly intracellular and extracellular enzyme activities were repressed almost equally.

In our normal experimental procedure the culture medium, together with the added extracts were autoclaved. It was important to determine whether this introduced an artefact into the results. Cucumber extracts were therefore added directly to sterile culture medium and growth and laccase formation followed. Under these conditions the same inhibition of laccase formation was observed, but growth of the fungus was also reduced slightly, but not below that of the controls (Table 6). Even the addition of slices of cucumbers to the culture medium resulted in inhibition of laccase formation under inducing conditions. From the above it is clear that cucumbers contain a compound or a number of compounds which inhibit laccase formation. The nature of these substances has not yet been determined. They are clearly heat stable, and water soluble. Preliminary experiments indicate that the inhibitory substances can be extracted by phosphate buffer at pH between 3.5 and 6.0. Non-polar solvents did not extract the active compounds. Experiments on characterization are continuing.

In additional experiments we observed that the cucumbers from which the extracts were made were quite resistant to attack by *Botrytis*, using the assay system previously described [5]. This resistance to attack also seemed to correlate with cucumber age. The level substances repressing laccase formation in cucumbers of different ages was compared. Since fruit size is an indicator of age we compared cucumbers 20 g in weight with those which were 60–80 g and 300 g per fruit. On a fresh weight basis the extracts from younger fruit repressed activity more than those from the medium and full sized fruits. This might suggest that the inhibitory substances are formed during early development of the fruit and become diluted as the fruit grows.

DISCUSSION

The repression of laccase formation by EDTA can probably be ascribed to chelation of copper, while it is likely that inhibition by the cucumber extracts is through a different mechanism. In the case of the EDTA repression, reversal is almost 100% if Cu^{2+} ions are added. It is therefore possible that treatment of host tissue prior to infection by the fungus results in a localized depletion of copper, which prevents laccase formation by the infecting fungus. We hypothesize that this may result in the inability of the mycelium to continue to penetrate the host, a step for which laccase is a necessary, but not sufficient, requirement. That copper is vital for laccase formation and that in its absence inactive enzyme is formed and excreted has been demonstrated for sycamore cells [12].

Table 5. Effect of cucumber solid residue or juice on formation of intra and extracellular laccase activity by *Botrytis cinerea* (additions to culture medium as in Table 4)

	Relative enzyme activity	
Growth medium	Extracellular	Intracellular
Malt + 2 inducers	100	100
Malt + 2 inducers '+solid residue'	7	10
Malt + 2 inducers +'juice'	6	11

Enzyme activity was measured in the culture medium after 17 days of growth or in an extract of a homogenate of the mycelium, 2–3 g/ml, clarified by centrifugation. Results are given per ml of culture medium or per ml of clarified extract. 100% enzyme activity was 3.3 μl O_2/ml/min. for the intracellular enzyme and 3.9 μl O_2/ml/min. for the extracellular enzyme.

Table 6. Effect of fresh solid residues on growth and laccase formation by *Botrytis cinerea*

Growth medium	Enzyme activity μl O_2/ml	Growth g/culture
Malt + 2 inducers	3.3	1.82
Malt + 2 inducers + 'solid residue'	0.32	1.62
Malt + 'solid residue'	0.16	1.64
Malt + 2 inducers + autoclaved 'solid residue'	0.25	2.6

Culture conditions and residue concentration as in Table 4. Activity measured after growth of culture for 12 days.

We have also observed partial correlations between the ability of cultivars of *Botrytis* to infect host tissue, its ability to form laccase and its resistance to fungicides of the carboxyimide type. The fungicide resistant strains had low infectivity (T. Katan, personal communication) and had lower laccase levels than the non-resistant strains. Additional circumstantial evidence comes from our observation that *B. squamosa*, which is very limited in its host range, has a very low level of extracellular and intracellular laccase, 10% of that of *B. cinerea*, which has a very wide range of hosts (Bar Nun and Mayer, unpublished). Again this points to a possible causal relation between laccase formation and infectivity.

The ability of fractions from cucumbers to repress laccase formation, the resistance of certain cucumber cultivars to infection and the presence in them of repressing compounds points in the same direction. We would like to suggest that susceptible varieties may lack or have a lower level of the laccase formation repressing compounds than the resistant varieties. Proof for these hypotheses is still lacking, and the inhibitory compounds must be isolated and identified. The results hold out some hope for devising new means to combat the infection by *Botrytis*.

EXPERIMENTAL

Botrytis cinerea was grown on a medium consisting of 2% malt extract, containing 0.01 M K-Pi–citrate buffer pH 3.5. To this medium citrus pectin, 1.6% was added before autoclavisation and gallic acid added as a solid to give a concentration of 0.1%, after sterilization. The flasks were then inoculated. Extracts of cucumber or other compounds were added to this medium. In the case of solid residues they were added to the medium, while in the case of the juices, they replaced part of the water used to make up the medium. In most of the experiments the fungus was grown in 500 ml Erlenmeyer flasks, containing 130 ml culture medium, without shaking, at 20° with weak incandescent light, as previously described [1–4].

Cucumbers were ground in a juice extractor, which separated a liquid phase and the solid residues. These were used as source for the experiments on inhibition by cucumber fractions.

Enzyme activity was followed using an oxygen electrode, with 10 mM quinol as the substrate, at pH 4.7, and 25°. Cucumbers were infected, after surface sterilization with hypochlorite, 2% as previously described [5]. When seedlings were used to test infection they were sprayed with the solutions of EDTA and the leaves then infected with a drop of suspension of the fungus, after locally injuring the leaves by slight scratching. The seedlings had 4–6 primary leaves. In the experiments on properties of the inhibitory compounds present in cucumber extracts, the fractions were extracted with solvents, the solvent removed by flash evapn and the residues dissolved in dist. H_2O before adding them to the culture medium.

Acknowledgement—This work has been supported in part by a grant from the Ernst David Bergmann Fund for Science.

REFERENCES

1. Gigi, O., Marbach, I. and Mayer, A. M. (1980) *Phytochemistry* **19**, 2273.
2. Gigi, O., Marbach, I. and Mayer, A. M. (1981) *Phytochemistry* **20**, 1211.
3. Marbach, I., Harel, E. and Mayer, A. M. (1983) *Phytochemistry* **22**, 1535.
4. Marbach, I., Harel, E. and Mayer, A. M. (1984) *Phytochemistry* **23**, 2713.
5. Marbach, I., Harel, E. and Mayer, A. M. (1985) *Phytochemistry* **24** 2559.
6. Mayer, A. M. and Harel, E. (1979) *Phytochemistry* **18**, 193.
7. Mayer, A. M. (1987) *Phytochemistry* **26**, 11.
8. Nicole, M., Geiger, J. P and Nandris, D. (1986) *Physiol. Mol. Plant Pathol.* **28**, 181.
9. Worrall, J. J., Chet, I. and Hutterman, A. (1986) *J. Gen. Microbiol.* **132**, 2527.
10. Dubernet, M. Riberau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 191.
11. Paoletti, S., Cesaro, A., Delben, F. and Ciana, A. (1986) in *Chemistry and Function of Pectins*, (Fishman M. L. and Jen, J. J., eds) American Chemical Society Symposium No. **310**.
12. Bligny, R., Gaillard, J. and Douce, R. (1986) *Biochem. J.* **237**, 583.