Phytochemistry, Vol. 24, No. 11, pp. 2559–2561, 1985.
Printed in Great Britain.

0031–9422/85 $3.00 + 0.00

PECTIN, A SECOND INDUCER FOR LACCASE PRODUCTION BY *BOTRYTIS CINEREA*

IRITH MARBACH, EITAN HAREL and ALFRED M. MAYER

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

(*Received* 22 *November* 1984)

Key Word Index—*Botrytis cinerea*; fungi; laccase; pectin; induction; pathogenicity.

Abstract—Pectin acts as a second inducer of extracellular laccase formation by *Botrytis cinerea*, in the presence of a phenolic substance as a first inducer, but pectin alone fails to induce enzyme formation. The possible advantages of this mechanism for the fungus during the process of infection and overcoming host resistance are discussed.

INTRODUCTION

In a number of papers [1–4] we have shown that *Botrytis cinerea* secretes laccase into the growth medium. The nature and properties of the laccases produced was determined by the phenolic inducer and we suggested that enzyme secretion might be related to phytopathogenicity [4]. In order to test this hypothesis further we examined whether metabolites of the plant cell wall might be related to laccase induction. Elicitors of phytoalexin formation are at least in some cases derivatives of pectins [5]. Since *Botrytis* excretes polygalacturonases and pectin methylesterases [6] the effect of pectin on laccase formation and excretion by *Botrytis* was studied in order to test whether the breakdown products of the plant cell wall might serve as signals for laccase formation. In the following we report that this is indeed the case.

RESULTS AND DISCUSSION

In order to test the effect of pectin on laccase formation and excretion by *Botrytis cinerea*, the fungus was grown in the presence of malt extract and phosphate citrate buffer pH 3.5 [3] to which either gallic acid (1 g/l.) or various concentrations of pectin or both were added at various times (Fig. 1 and Table 1). It can be seen from the results that the simultaneous addition of pectin and gallic acid greatly enhances laccase excretion. Prior partial degradation of the pectin, using Pectinol R-10 to reduce viscosity to 39%, did not change the effectiveness of the pectin. Time of addition of pectin was crucial. Only if the pectin was added at the onset of growth was its effect fully achieved (Table 1).

The effect of pectin might be due to an impurity or due to a change of the cation concentration of the growth medium. The possibility of an impurity could be ruled out as pectin repurified by ethanol precipitation was as effective as unpurified material. The cation content of a pectin solution (10 g/l.) was determined. Such a solution contained only small amounts of Cu^{2+}, Ca^{2+}, Mg^{2+} and K^+, compared to the normal growth medium, but addition of the pectin resulted in the doubling of the Na^+ concentration of the medium. However Na^+ was found to be neither an activator of laccase nor involved in its

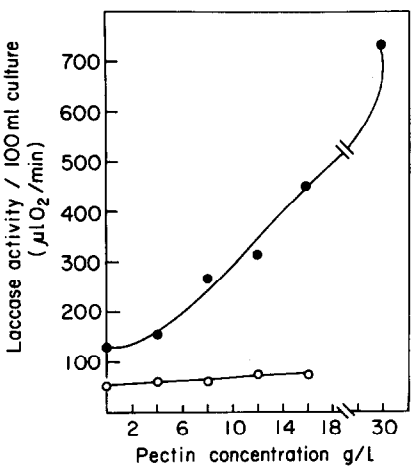

Fig. 1. Effect of pectin concentration on level of extracellular laccase in growth medium of *Botrytis cinerea*. ●——●, Gallic acid 1 g/l. + pectin; ○——○, pectin only.

Table 1. The effect of time of adding pectin (16 g/l.) and gallic acid (1 g/l.) on extracellular laccase formation by *Botrytis cinerea*

Time of adding after inoculation of:		Total laccase activity as % of formation when gallic acid added at zero time
Gallic acid	Pectin	
0	—	100
—	0	54
—	—	37
0	0	315
3	0	180
7	0	74
0	3	153
0	7	93

structure. Addition of Na^+ to the growth medium, to concentrations equivalent to that of medium plus pectin, slightly reduced laccase production. Pectin did not form a complex with gallic acid. UV spectra of gallic acid and pectin were simply additive. Neither fungal growth nor total protein excretion by the fungus were affected by addition of pectin. Thus it appears that pectin or its breakdown products act as a second inducer of laccase formation by *Botrytis*. One of the end products of pectin breakdown, galacturonic acid, did not enhance laccase production. The idea that pectin acts as a secondary inducer is supported by the fact that the isoelectric focussing pattern of the enzyme formed in the presence of pectin and gallic acid is identical to that produced when only gallic acid is present. Since, during infection, the fungus penetrates between the cells of the host and, by secreting polygalacturonases, causes breakdown of the middle lamella [7], products of pectin breakdown would be one of the chemical entities the pathogen encounters during infectivity. At the same time many host plants produce phytoalexins which are at least partly involved in reducing the progress of the pathogen [5]. Frequently these are phenolic in nature. In addition, phenolic compounds have also been considered as possible antifungal substances involved in the host response to infection. One might, therefore, expect that the pathogen would develop enzyme systems which are capable of inactivating such phytoalexins or phenolics [8]. Laccase might be one such enzyme. If this is the case the progress of the fungus through infected tissue should be accompanied by laccase production. We tested this idea by assaying the laccase activity in host tissue as a function of distance from the region of infection. The test system used was cucumber fruit, which is devoid of endogenous polyphenol oxidase. A small area of peel was removed (*ca* 1 cm^2) and the exposed surface of the fruit was inoculated at a central point with a suspension of *Botrytis*. After suitable periods of incubation the fruit was sliced and laccase measured as a function of distance from the point of infection. Colonization of the tissue was closely accompanied by laccase secretion. It was necessary to demonstrate that the laccase in the tissue originated in the fungus and not in the host. Mechanical injury of the tissue without infection did not result in enzyme formation. Infection of the tissue by a pathogen which does not secrete laccase, e.g. *Sclerotinia sclerotium*, did not result in the appearance of enzyme in the host tissue. In contrast, infection by 11 different isolates of *Botrytis* always resulted in laccase secretion. Thus laccase formation is an integral part of the infective process.

On the basis of these experiments it might be suggested that the fungus requires two signals for maximal laccase production. One signal is provided by phenolic compounds, which are potentially toxic. The second signal is provided by decomposition products of pectin which also signal the extent of breakdown of host tissues. It would thus be expected that extracts of healthy cucumber fruits contain the necessary compounds to induce laccase formation by *Botrytis* on growth media. Indeed an autoclaved and filtered homogenate of cucumbers served as a good inducer of laccase formation by *Botrytis* when added to normal growth medium without any other inducer.

Botrytis is a pathogenic fungus which attacks a wide range of hosts [9]. Such a pathogen might be expected to control the secretion of enzymes very carefully, in response to the precise nature of the host plant. Our previous work appeared to indicate that the fungus adjusted the molecular structure of the extracellular laccase to the pH of the host and the nature of the phenolics present in it. This work indicates that the amount of enzyme is also controlled, perhaps by the amount of plant cell wall material degraded during attack. In previous reports on induction of laccase in various fungi [10–13] there was no indication of control of its extracellular level by two quite distinct inducers. It should be recalled that the enzyme laccase is a glycoprotein [4] and this property probably contributes to its stability outside the fungal cell, as has been suggested for extracellular fungal hydrolases [14]. These features suggest that enzyme activity within infected tissue can be closely regulated by the proposed two-signal system.

EXPERIMENTAL

Botrytis cinerea was cultured as previously described [15] in 500 ml flasks containing 130 ml medium. The pectin used was apple pectin having 6% methoxy group obtained from B.D.H. Pectinol R-12, used to partially degrade the pectin, was obtained from Rohm and Haas, partially purified as described in ref. [16].

Laccase was extracted from cucumber fruit by homogenization, filtration through cheese cloth and centrifuging for 15 min at 1000 *g*. The supernatant was used as source of enzyme.

Estimation of enzyme activity was with an oxygen electrode using quinol, 10 mM, as substrate [15]. Cation concn (Ca^{2+}, Mg^{2+}, Cu^{2+}, K^+ and Na^+) in the growth medium and soln of pectin were determined by atomic absorption spectroscopy.

Infection of cucumber fruits. Cucumber fruits were surface sterilized with 2% Na hypochlorite soln for 20 min and then rinsed with sterile H_2O. Part of the peel was removed and the exposed area of the fruit was inoculated with a suspension of *Botrytis* spores and some mycelium. Infection with *Sclerotinia* was done in the same way using a mycelial suspension.

Acknowledgement—This work was supported in part by a grant from the National Research Council for Research and Development of Israel.

REFERENCES

1. Dubernet, M., Ribereau-Gayon, P., Lerner, H. R., Harel, E. and Mayer, A. M. (1977) *Phytochemistry* **16**, 191.
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. Darvill, A. G. and Albersheim, P. (1984) *Ann. Rev. Plant Physiol.* **35**, 243.
6. Marcus, L. and Schejter, A. (1983) *Physiol. Plant Pathol.* **23**, 1.
7. Verhoeff, K. (1980) in *The Biology of* Botrytis (Coley-Smith, J. R., Verhoeff, K. and Jarvis, W. R., eds) p. 153. Academic Press, New York.
8. Mansfield, J. W. (1980) in *The Biology of* Botrytis (Coley-Smith, J. R., Verhoeff, K. and Jarvis, W. R., eds) p. 181. Academic Press, New York.
9. Jarvis, W. R. (1980) in *The Biology of* Botrytis (Coley-Smith, J. R., Verhoeff, K. and Jarvis, W. R., eds) p. 219. Academic Press, New York.
10. Wood, D. A. (1980) *J. Gen. Microbiol.* **117**, 339.

11. Petroski, R. J., Peczynska-Czoch, W. and Rosazza, J. R. (1980) *Appl. Environ. Microbiol.* **40**, 1003.
12. Haars, A. and Hutterman, A. (1983) *Arch. Microbiol.* **134**, 309.
13. Leonowicz, A. and Malinowska, M. (1982) *Acta Biochim. Polon.* **29**, 219.
14. Civas, A., Eberhard, R., LeDizet, P. and Petek, F. (1984) *Biochem. J.* **219**, 857.
15. Gigi, O., Marbach, I. and Mayer, A. M. (1980) *Phytochemistry* **19**, 2273.
16. Albersheim, P. (1966) *Methods in Enzymology*, Vol. 8, p. 628. Academic Press, New York.