Phytochemistry, Vol. 29, No. 3, pp. 787–791, 1990.
Printed in Great Britain.

0031-9422/90 $3.00+0.00

CUCURBITACINS PROTECT CUCUMBER TISSUE AGAINST INFECTION BY *BOTRYTIS CINEREA*

NURIT BAR-NUN and ALFRED M. MAYER

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

(*Received in revised form* 20 *July* 1989)

Key Word Index—*Botrytis cinerea*; cucurbitacin I; cucumber; infection.

Abstract—Extracts of *Ecballium elaterium* or cucurbitacin I were applied to cucumber fruits or plants or to cabbage leaves, prior to inoculation with *Botrytis cinerea*. This treatment prevented infection of the tissue, the infecting fungus being restricted to the site of infection. Infection was always accompanied by localized lignification. The protective effect was not due to the induction of lignification. It is suggested that the ability of cucurbitacin I to inhibit induction of laccase formation by *Botrytis* is responsible for its protective effect.

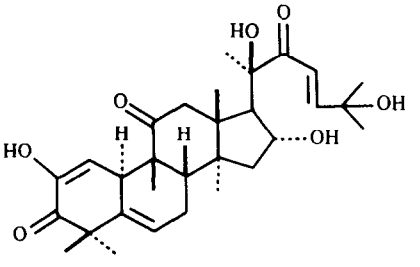
INTRODUCTION

The Cucurbitaceae contain tetracyclic triterpenoids, collectively referred to as cucurbitanes or cucurbitacins [1]. These are pharmacologically very active compounds. They have been shown to: act as kairomones, plant chemicals which are of benefit to the insect predator, e.g. by acting as attractants [2, 3], be generally toxic to mammalian cells [4], and be responsible for the bitter taste of cucumber fruit [5]. Their effect on fungi has hardly been investigated although inhibition of the growth of *Phytophthora cactorum* has been reported [6]. Their biological role in plants remains uncertain. We have recently shown that cucurbitacins I and D are inhibitors of the induction of extracellular laccase formation by cultures of *Botrytis cinerea* [7]. We hypothesized that laccase formation is part of the infective process by *Botrytis* and that prevention of laccase formation might afford protection of the host against the invading fungus [8]. We have considered the possibility that cucurbitacins might play a role (so far unreported), as antifungal agents in plant tissues and may have the same function as phytoalexins. This possibility has been studied by applying extracts of fruits of *Ecballium elaterium* (squirting cucumber), a ready source of cucurbitacins [1, 9, 10], or authentic cucurbitacin I, (**1**) to cucumber fruit or cabbage leaves prior to, or during, inoculation with *Botrytis*. The following describes some of the results, which support the concept of an antifungal role for these compounds.

RESULTS AND DISCUSSION

Infection by *Botrytis* of cucumber fruits pretreated with water or with a partially purified extract of *Ecballium elaterium* for 24 hr was followed for up to 10 days. Control fruits, four days after inoculation, showed considerable signs of infection, chlorosis and the onset of cell dissociation and eventual disintegration. In contrast the fruits pretreated with *Ecballium* extract were healthy, showed no signs of fungal development or of chlorosis. These experiments were repeated, but cucurbitacin I (**1**) (0.01%), was applied to the fruits during pretreatment. The results using the partially purified extract and the pure cucurbitacin were indistinguishable, both affording almost complete protection against infection over the test period, up to two weeks. Typical effects using the partially purified extract or cucurbitacin I are shown in Plate 1, (Figs 1–3). Similar experiments were carried out using the inner leaves of white cabbage as the test object, Plate 1 (Figs 4 and 5). Again it is quite clear that the pretreatment with cucurbitacin reduced the degree of infection very considerably. Protection was also observed in leaves of cucumber plants, pretreated with cucurbitacin. The leaves of control plants rapidly deteriorated, while those of pretreated leaves showed only injury at the inoculation site (data not shown).

By day 10, the control, inoculated fruit had virtually disintegrated. An indication of the degree of tissue disintegration could be obtained from the amount of extractable-free galacturonic acid resulting from cell wall degradation. This was followed as a function of the distance from the point of infection (Table 1). The cucumbers treated with the *Ecballium* extract or with cucurbitacin I showed only the background level of galacturonic acid, due to sugars present in the tissue reacting with dinitrosalicylic acid. This level was more or less constant throughout the fruit. In contrast, in the control fruit the level of free galacturonic acid rose sharply due to cleavage



(**1**)

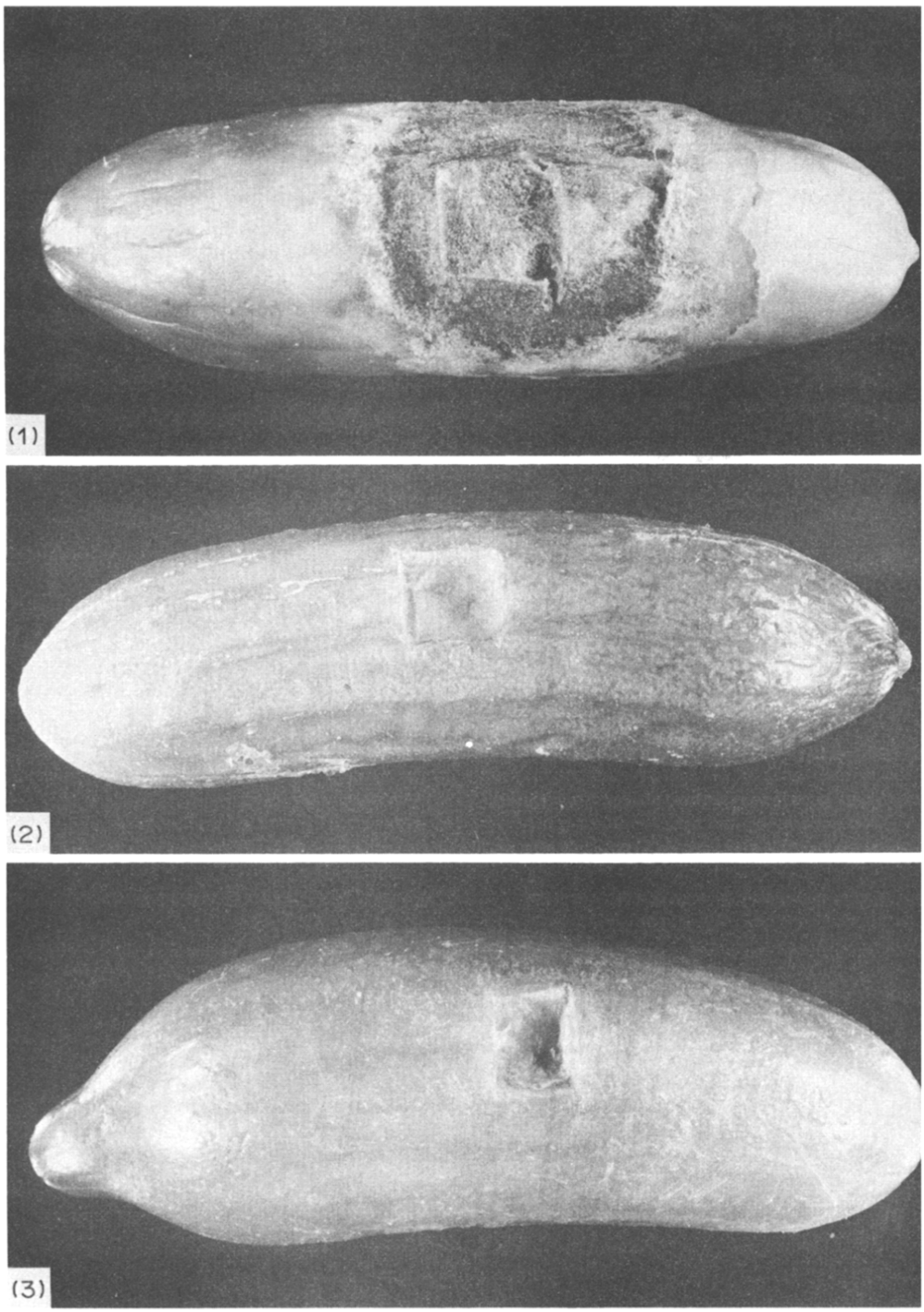


Fig. 1. Cucumber fruit infected with *Botrytis cinerea*. Note spread of fungus, 120 hr after inoculation.

Fig. 2. Cucumber fruit pretreated with *Ecballium* extract prior to inoculation. Note absence of fungal infection. Photograph 120 hr after inoculation.

Fig. 3. Cucumber fruit pretreated with cucurbitacin prior to inoculation. Photograph 120 hr after inoculation.

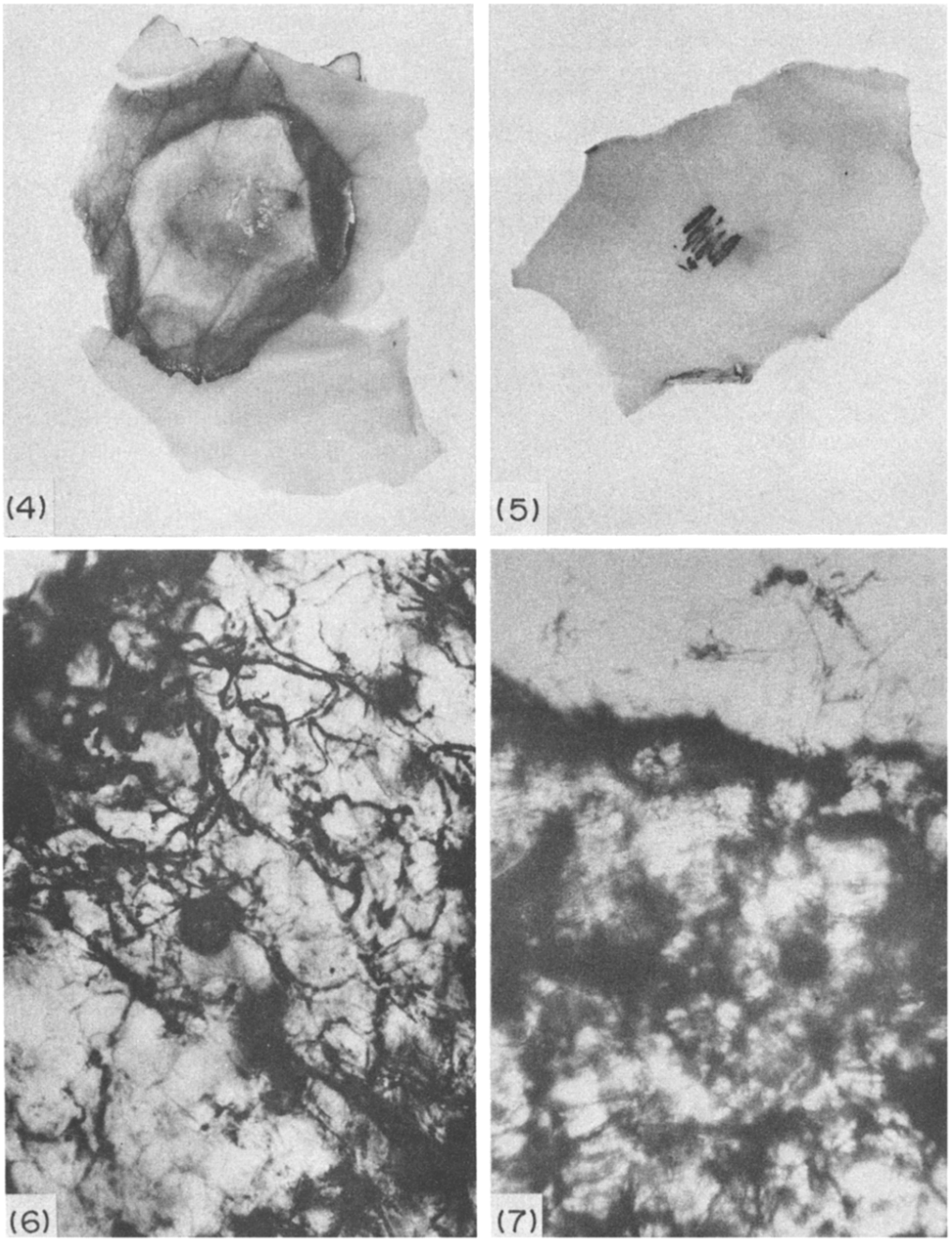


Fig. 4. Cabbage leaf infected with *Botrytis*. Photographed 120 hr after inoculation. Fungus has spread over entire leaf area.

Fig. 5. Cabbage leaf pretreated with *Ecballium* extract prior to inoculation with *Botrytis*. Photographed 120 hr after inoculation. Note darkening only at site of injury.

Fig. 6. Section through surface of infection well of control cucumber fruit, 96 hr after inoculation. Hyphae present throughout the tissue.

Fig. 7. Section through infection well of cucumber fruit pretreated with cucurbitacin prior to inoculation. Photographed, 96 hr after inoculation. Note hyphae present only on surface.

Table 1. Free galacturonic acid content of cucumber fruit at various distances from point of infection

Distance (cm) from point of infection	Control	Inoculated	Inoculated + *Ecballium* extr. pur.	Inoculated + cucurbitacin
2	32.5 (2.5)	126 (6.5)	35	26 (1.0)
4	32.0 (3.2)	97.5 (17.5)	35	26 (1.0)
6	29.5 (3.9)	72 (12)	35	25.5 (1.0)

Results seven days after inoculation, as mg galacturonic acid/g. fr.wt fruit. Results as mean of three replicates, S.D. in parentheses. Data in column (4), *Ecballium* extract, were repeated only once because values were undistinguishable from controls and were not pooled with the subsequent column, cucurbitacin.

of polysaccharide polymers, pectins in the cell walls indicating tissue disintegration, due to extra-cellular pectin degrading enzymes excreted by the fungus. Such pectin-degrading enzymes, secreted by *Botrytis* have been described by a number of authors [11–15].

The response of cucumber fruit to infecting fungi has been reported by many authors. One of the characteristic reactions is what appears to be lignification at the site of infection [16, 17]. Such lignification has been considered to be part of the protective or resistance mechanism of plants to many fungi [18–22]. The protection resulting from the application of cucurbitacins was not an indirect one, caused by the stimulation of lignification alone as shown by comparisons of cucumber fruits treated with water, or with cucurbitacin, with or without infecting *Botrytis*. Such fruit was hand sectioned and stained for lignification near the site of infection, (Table 2). The presence or absence of laccase in the host tissue was also examined after 96 hr. It can be seen that neither wounding alone nor application of cucurbitacin induced any marked degree of lignification. In contrast, infection by *Botrytis* alone resulted in marked lignification, which, however, did not prevent penetration and spread of the fungus through the lignified layer (Plate 1, Figs 6 and 7). In the case of cucurbitacin-treated fruits infected with *Botrytis*, lignification also occurred, but in this case the fungus did not penetrate the lignified layer.

The mycelium from treated fruit, although it did not develop at the site of infection, was not killed. Mycelium could be scraped from the infection wells and reinoculated into culture medium. This mycelium grew rapidly and under inducing conditions was able to produce laccase. Laccase was also readily detected in fruit in which infection and tissue disintegration proceeds (Table 2),

Table 2. Appearance of apparent lignification at site of infection of cucumber fruit with or without infection by *Botrytis*, treated or non-treated with crude or partially purified *Ecballium* extract or with cucurbitacin I.

	Presence after						
	24 hr		48 hr		96 hr		
Treatment	Lignin*	Fungus†	Lignin	Fungus	Lignin	Fungus	Laccase
Control	+/−	−	+	−	+	−	−
Inoculated	++	+	++	+	++	+	+
Ecballium ext.	+	−	+	−	+	−	−
Inoculated + *Ecballium* ext.	+	−	+	−	+	−	−
Pur. *Ecballium* ext.	−	−	−	−	+/−	−	−
Inoculated + pur. *Ecballium* ext.	+/−	−	+	−	++	−	−
Curcurbitacin	−	−	+/−	−	+/−	−	−
Infected + cucurbitacin	+/−	−	+	only on surface	+	only on surface	−

Cucumbers were treated as described in Experimental, tissue removed, sectioned and stained and the degree of lignification estimated. Experiments were repeated on four different samples of fruit, over a period of several weeks.

*Lignin: +/− very little lignification; + lignification of a layer of at least two cells thick; + + marked lignification, several cell layers thick.

†Fungus: mycelium in the cucumber tissue: present +; absent −.

using either the direct assay for laccase as described by Marbach *et al.* [23] or by employing the histochemical nitrocellulose direct tissue blotting techniques described by Spruce *et al.* [24], using syringaldazine as the substrate.

EXPERIMENTAL

Cucumber fruits and white cabbage were purchased at a local supermarket. *Botrytis cinerea* was grown as previously described by Marbach *et al.* [23, 25]. Mycelium used for inoculating the plant tissue was grown in the presence of malt only, 20 g/l in 0.1 KPi/citrate buffer, without addition of inducers of laccase activity. *Ecballium elaterium* fruit were collected in the gardens of the Hebrew University. Cucurbitacins were extracted and partially purified as described by Rehm *et al.* [10].

Infection. Cucumbers and cabbage leaves were surface sterilized with ethanol. A small well, 10 × 5 mm, 5 mm deep, was cut in the centre of the cucumber fruit [25]. Into this well a small amount, 0.2 ml, of sterile dist. H_2O or test solution was introduced, and left there for 24 hr before inoculation. In the case of partially purified *Ecballium* extract, as described in [7], or pure cucurbitacin (0.2 mmol), the pretreatment was with a 25% alcoholic soln and the controls were treated only with 25% EtOH. A suspension of mycelium was then introduced into the wells and the progress of the infection followed. Cabbage leaves were injured by scratching with fine forceps, the test solution placed on the point of injury and the mycelium again introduced after 24 hr. During pretreatment and following inoculation the plant tissue was kept in closed glass crystallizing dishes in which a high relative humidity, near 100%, was maintained. The dishes were kept at 22–24° in dim light, from incandescent and fluorescent light, between 3.5–5.5 μ Einstein/m^2/sec. The experiment with the different treatments was repeated × 10 on different samples of cucumbers. The photograph shows a typical and reproducible result.

Tissue disintegration. When breakdown of cells was followed, slices from the fruit, cut at various distances from the site of inoculation were homogenized and their galacturonic acid content was determined by the 3,5-dinitro salicylic acid procedure [26].

Light microscopy and staining. At various times after pretreatment or inoculation, tissue near the initial site of infection was removed and hand sectioned for staining with 0.1% trypan blue in 45% HOAc followed by rinsing and fixing in lacto-phenol. The same sections were also stained for apparent lignification using either saturated aq. phloroglucinol, followed by addition of concd HCl or a saturated acidic solution of aniline chloride. Generally staining with the two stains coincided precisely. If the same cells were stained red with phloroglucinol or yellow with aniline chloride, we took this to indicate apparent lignification.

Acknowledgement—Our thanks are due to Professor D. Lavie for providing us with cucurbitacin. I.

REFERENCES

1. Lavie, D. and Glotter, D. (1971) *Progr. Chem. Nat. Prod.* **29**, 308.
2. Metcalf, R. L., Metcalf, R. A. and Rhodes, A. M. (1980) *Proc. Natl Acad. Sci.* **77**, 3769.
3. Ferguson, J. E. and Metcalf, R. A. (1985) *J. Chem. Ecol.* **11**, 311.
4. Stoewsand, G. S., Jaworski, S., Shannon, S. and Robinson, R. W. (1985) *J. Food Protect.* **48**, 50.
5. Gorski, P. M., Jaworski, A., Shannon, S. and Robinson, R. W. (1986) *HortSci.* **21**, 1034.
6. Nes, W. D. and Patterson, G. W. (1981) *J. Nat. Prod.* **44**, 215.
7. Bar-Nun, N. and Mayer, A. M. (1989) **28** *Phytochemistry* **28**, 1369.
8. Bar-Nun, N., Tal-Lev, A., Harel, E. and Mayer, A. M. (1988) *Phytochemistry* **27**, 2505.
9. Lavie, D. and Willner, D. (1958) *J. Am. Chem. Soc.* **80**, 710.
10. Rehm, S., Enslin, P., Meeuse, A. D. J. and Wessels, J. H. (1957) *J. Sci. Food Agric.* **8**, 679.
11. Urbanek, H. and Zalewska-Sobczak, J. (1975) *Biochim. Biophys. Acta* **377**, 402.
12. Magro, P., Di Lenna, P., Marciano, P. and Pallavicini, C. (1980) *J. Gen. Microbiol.* **120**, 105.
13. Marcus, L. and Schejter, A. (1983) *Physiol. Plant Pathol.* **23**, 1.
14. Heale, J. B. (1987) in *Proc. E. C. Experts group.*, Portoferraio, (Cavallora, R., ed.) p. 277.
15. Heale, J. B. and Movahedi, S. (1989) *Proc. 4th International Symp. Oenology*, Bordeaux (in press).
16. Hammerschmidt, R. and Kuc, J. (1982) *Physiol. Plant Pathol.* **20**, 61.
17. Dean, R. A. and Kuc, J. (1987) *Physiol. Mol. Plant Pathol.* **31**, 69.
18. Ride, J. P. (1975) *Physiol. Plant Pathol.* **5**, 125.
19. Ride, J. P. *Ann. Appl. Botany* **89**, 302.
20. Pearce, R. B. and Ride, J. P. (1980) *Physiol. Plant Pathol.* **16**, 197.
21. Friend, J. (1976) *Proc. Phytochem. Soc.* **13**, 291.
22. Friend, J. (1985) *Ann. Proc. Phytochem. Soc. Eur.* **25**, 367.
23. Marbach, I., Harel, E. and Mayer, A. M. (1983) *Phytochemistry* **22**, 1535.
24. Spruce, J., Mayer, A. M. and Osborne, D. J. (1987) *Phytochemistry* **26**, 2901.
25. Marbach, I., Harel, E. and Mayer, A. M. (1985) *Phytochemistry* **24**, 2559.
26. Bernfeld, P. (1955) in *Methods in Enzymology* I (Colowick, S. P. and Kaplan, N. O. eds), pp. 149–158. Academic Press, New York.