Induction of *Nectria galligena* Mutants Resistant to Benzoic Acid and Study of Their Aggressiveness Towards Immature Apples

By JEAN-MARC SENG,† PATRICK SAINDRENAN* AND GILBERT BOMPEIX

Laboratoire de Pathologie Végétale, Université P. et M. Curie (Paris VI),
Tour 53 – 4ème étage – 4 Place Jussieu, 75230 Paris Cedex 05, France

(Received 16 January 1985; revised 9 April 1985)

The isolation of *Nectria galligena* mutants resistant to benzoic acid (BA), the phytoalexin of immature apples, is reported. Those in which pathogenicity on mature apples was similar to the wild-type were inoculated in immature fruits. One mutant resistant to BA was more aggressive than the wild-type in young apples at the end of their maturation cycle. Aggressiveness of this mutant and the wild-type could be increased by growing the fungus before inoculation into the fruit on a medium supplemented with BA. Amounts of BA in mutant-infected and wild-type-infected tissues were similar. We conclude that BA is involved in the resistance of immature apples infected by *N. galligena*.

INTRODUCTION

There is still considerable controversy about the role of phytoalexins in plant resistance to fungal pathogens. In several host-parasite interactions, a strong correlation has been established between the amounts of phytoalexins and resistance to the pathogen (Yoshikawa *et al.*, 1978; De Wit & Flach, 1979; Bailey *et al.*, 1980; Mansfield, 1982). However, little is known about the biological activity of these compounds *in vivo* during the expression of resistance (Mayama *et al.*, 1982; Moesta & Grisebach, 1982). Consequently, the hypothesis that phytoalexins are only by-products of pathogenesis and do not play a role in resistance cannot be ruled out (Ward, 1983). In this paper, we report the isolation of mutants of *Nectria galligena* resistant to benzoic acid (BA), the phytoalexin of immature apples (*Malus pumila*), and present a study of their aggressiveness compared to that of the wild-type strain.

METHODS

Fungus. *Nectria galligena* wild-type strain (isolate 7801) was isolated from a naturally-infected apple. After growth from a single microconidium, the pathogen was cultivated on malt agar medium comprising (per litre): maltea Moser 10 g (Moser, Lyon, France), Difco Bacto Agar 15 g.

Fruits. Immature fruits of the apple cultivar Smoothie were harvested 106 d after full blossom from an experimental orchard in Northern France and kept in aerated plastic bags at 4 °C. Mature fruits of the Golden Delicious cultivar were obtained from a store in Paris.

Inoculation. Apples were surface-sterilized in 8% (w/v) calcium hypochlorite. Wounds 5 mm in diameter and 3 mm deep were made at the equator with a cork-borer. A plug (5 mm in diameter) was taken from the margin of the growing mycelium. The mycelium was sliced off the plug with a scalpel and placed at the bottom of the wound. A plug of fresh medium was then used to fill the hole. Mycelial progression could be easily observed because of the radial growth of *N. galligena* at the surface of the fruit. The increase in diameter of the lesion was recorded and used as an estimate of pathogenicity and aggressiveness.

† Present address: Laboratoire de Cryptogamie, Université de Paris-Sud, Centre d'Orsay, Bâtiment 400, 91405 Orsay Cedex, France.

Abbreviation: BA, benzoic acid.

Induction, selection and characterization of mutants. UV mutagenesis. A fungal thallus, grown for 3 d on a malt agar medium covered with a cellophane membrane, was exposed to UV light at 254 nm (750, 1000 and 1250 J m⁻²). Immediately after UV treatment, the membrane was transferred in semi-darkness to malt agar medium supplemented with 250 µg BA ml⁻¹ and buffered at pH 3.5 with 0.1 M-citric acid/0.2 M-phosphate. Petri dishes containing this screening medium were then placed in darkness for 15–20 d.

X-ray mutagenesis. A Petri dish supporting a 3-d-old thallus grown as described above was covered with an aluminium screen 5 mm thick and exposed to X-rays (10000 and 20000 rad) from a Baltographe 5–50 kV/20 mA, adjusted to 10 mA and 50 kV. After irradiation, the membrane was transferred to the screening medium and placed at 23 °C in the light for 15–20 d.

NTG mutagenesis. A cellophane membrane supporting a 3-d-old thallus grown as described above was transferred to screening medium which was also covered with a cellophane membrane; 0.1 ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) in aqueous solution (150 µg ml⁻¹ and 300 µg ml⁻¹) was introduced between the two membranes, which were then placed at 23 °C in the light for 15–20 d.

From 8 to 15 d after mutagen treatments, depending on the mutagenic agent used, fast growth rate sectors were initiated from the margin of the culture. The hyphal tips of these sectors were isolated and allowed to grow on fresh medium in the presence, and in the absence, of BA. Sectors which grew well were retained and tested against the wild-type at pH 3.5 and 4.5 and at different BA concentrations.

Extraction and estimation of BA from apple tissue. BA was extracted in ethyl acetate (Brown & Swinburne, 1971) and quantified by capillary-gas-liquid chromatography (Noble & Drysdale, 1983).

RESULTS

X-ray and UV mutagenesis gave rise to mutants whose growth rates were 30–50% higher than that of the wild-type, whether the medium was supplemented with BA or not. These mutants were as sensitive to BA as the wild-type (results not shown). Five mutants, designated NTG 1 to 5, were obtained by NTG (300 µg ml⁻¹) mutagenesis. When compared to the wild-type, their lag-phase at pH 3.5 and 4.5 on a medium supplemented with BA was shorter. At 200 µg BA ml⁻¹ and pH 4.5, their lag-phase was 1–2 d while that of the wild-type was 2–3 d (Figs 1 and 2). At all BA concentrations, the growth rates of the strains were similar but NTG 2 and 5 tolerated higher concentrations of BA (150 µg ml⁻¹ and 300 µg ml⁻¹ at pH 3.5 and 4.5 respectively) than the wild-type (75 µg ml⁻¹ and 200 µg ml⁻¹ at pH 3.5 and 4.5 respectively). For further studies, we selected NTG 2 and NTG 5, and two fast growth rate mutants (CR 3 and CR 7) whose morphology on malt agar medium did not differ from that of the wild-type. Pathogenicity of the different mutants was maintained through inoculation in mature apples. Only NTG 5, CR 3 and CR 7, which caused a rot similar to that of the wild-type, were retained and were inoculated in immature fruits to test their aggressiveness.

Mutants CR 3 and CR 7 exhibited the same behaviour as the wild-type when inoculated at the equator of immature apples. In contrast, mutant NTG 5 seemed to be more aggressive than the wild-type when the strains were cultivated on malt agar medium (Table 1). In addition, a more abundant mycelium was observed in the wound colonized by the mutant. When the strains were cultivated on malt agar medium containing BA, the diameters of lesions subsequently caused by both strains were 3–4 mm larger than those observed when the fungi were cultivated without BA (Table 1). This was observed 10 and 19 d after inoculation. When the diameters of lesions caused by the two strains were compared, NTG 5 was again more aggressive than the wild-type.

BA was extracted from lesions infected by NTG 5 and the wild-type cultivated on the two media described above (Table 1). BA content was always greater in the infected area at day 19 than at day 10, whatever the culture medium; however, differences observed in BA concentrations between NTG 5 and the wild-type were slight.

DISCUSSION

Aggressiveness of mutant NTG 5 and that of the wild-type were compared by measuring the lesion diameter at the surface of the fruit. It has been shown (Swinburne, 1971) that *N. galligena* can cause cell death well in advance of the hyphal tip, so that the diameter of the necrotic zone visible at the surface of the fruit does not necessarily reflect precisely the volume of tissues colonized by the fungus. However, the comparison between the two strains remains valid.

Fig. 1. Radial growth of *N. galligena* wild-type strain on malt agar supplemented with BA at the concentrations ($\mu\text{g ml}^{-1}$) indicated on the figure. (a) pH 3.5, (b) pH 4.5 except (▲) pH 5.5.

Fig. 2. Radial growth of *N. galligena* mutant NTG 5 on malt agar supplemented with BA at the concentrations ($\mu\text{g ml}^{-1}$) indicated on the figure. (a) pH 3.5, (b) pH 4.5.

Table 1. *Benzoic acid concentrations and lesion diameters in immature apples inoculated with NTG 5 and wild-type strains of N. galligena grown on different culture media*

Culture medium	Time after inoculation (d)	Strain	Benzoic acid concn [$\mu\text{g (g fresh wt apple)}^{-1}$]	Lesion diameter* (mm)
Malt agar	10	Wild-type	162 $\pm$ 33	1.0 $\pm$ 0.4
		NTG 5	205 $\pm$ 30	2.2 $\pm$ 0.3***
	19	Wild-type	480 $\pm$ 31	4.5 $\pm$ 0.4
		NTG 5	580 $\pm$ 39	6.4 $\pm$ 0.6***
Malt agar supplemented with BA (200 $\mu\text{g ml}^{-1}$, pH 4.5)	10	Wild-type	137 $\pm$ 33	4.8 $\pm$ 0.8
		NTG 5	270 $\pm$ 25	6.5 $\pm$ 1.0***
	19	Wild-type	609 $\pm$ 51	6.4 $\pm$ 0.6
		NTG 5	630 $\pm$ 32	9.4 $\pm$ 0.9**

* The lesion diameter was determined by subtracting the diameter of the wound made by the cork-borer from that of the whole lesion. Each value is the mean of 15 diameters measured on 15 fruits infected per experiment. Each experiment was repeated three times. All values for the mutants were compared using a *t*-test for paired values to the respective wild-type strain grown on the same fruits. ** Significant at $P = 0.01$, *** significant at $P = 0.001$.

The wild-type strain exhibited a more aggressive behaviour towards young fruits when grown on malt agar containing BA than when grown on malt agar without BA (Table 1). This would indicate that adaptation to BA occurs *in vitro* and increases the aggressiveness of the wild-type. Although the mechanism of this phenomenon is not known, it suggests that BA is a resistance factor of immature apples. Mutant NTG 5 exhibited a more aggressive behaviour than the wild-type when inoculated in young fruits at the end of their maturation cycle. This was observed

whatever the culture medium of the fungus before inoculation. With an inoculum grown on malt agar containing BA, the diameters of lesions NTG 5 were greater at day 10 and 19 than those obtained with inocula grown on malt agar without BA. This would indicate that the adaptation observed with the wild-type still occurs with the BA resistant mutant.

It has been shown that proteases of *N. galligena* are the elicitors of BA in immature apples (Swinburne, 1975). However, the synthesis of proteases by NTG 5 compared to the wild-type strain has yet to be examined in detail. At day 19, the growth of NTG 5 and the wild-type strain occurred at nearly 500 µg BA (g fresh weight)⁻¹. It is surprising that the two strains develop a rot in the presence of such high amounts of BA but one explanation could be that BA itself is not in contact with the fungus. BA is a weak lipophilic acid, and thus permeates membranes very easily in the undissociated form (Warth, 1977; Albert, 1981), but it would dissociate in the cytoplasm, which is at pH 5.0–5.5 in both plant and fungus, into the benzoate anion, which might not be active against the fungus (Krebs *et al.*, 1983). If the BA is in contact with the fungus, three hypotheses can be advanced to explain the growth of the mycelium in such an environment: (1) the biosynthesis of BA occurs late so that the phytoalexin accumulates behind the hyphal tips in the macerated area; (2) a mechanism exists by which the fungus tolerates high levels of BA; (3) BA concentrations alone do not perfectly reflect the resistance of the fruit, and resistance would have been better estimated by relating the concentration of BA to the amount of fungus in infected tissues. At this stage in our work, it is difficult to favour one of these hypotheses. However, our results suggest a role for BA in apple tissue resistance.

The authors are very grateful to Professor J. Chevaugnon and Dr M. J. Daboussi for discussion of the work and for suggestions relative to the preparation of the manuscript. We also thank J. Reams for help with the English.

REFERENCES

- ALBERT, A. (1981). Ionization. In *Selective Toxicity. The Physico-chemical Basis of Therapy*, 6th edn, pp. 330–382. Edited by A. Albert. London: Chapman and Hall.
- BAILEY, J. A., ROWELL, P. M. & ARNOLD, G. M. (1980). The temporal relationship between infected cell death, phytoalexin accumulation and the inhibition of hyphal development during resistance of *Phaseolus vulgaris* to *Colletotrichum lindemuthianum*. *Physiological Plant Pathology* **17**, 329–339.
- BROWN, A. E. & SWINBURNE, T. R. (1971). Benzoic acid: an antifungal compound formed in Bramley's Seedling apple fruits following infection by *Nectria galligena* Bres. *Physiological Plant Pathology* **1**, 469–475.
- DE WIT, P. J. G. M. & FLACH, W. (1979). Differential accumulation of phytoalexins in tomato leaves but not in fruits after inoculation with virulent races of *Cladosporium fulvum*. *Physiological Plant Pathology* **15**, 257–267.
- KREBS, H. A., WIGGINS, D., STUBBS, M., SOLS, A. & BEDOYA, F. (1983). Studies on the mechanism of the antifungal action of benzoate. *Biochemical Journal* **214**, 657–663.
- MANSFIELD, J. W. (1982). The role of phytoalexins in disease resistance. In *Phytoalexins*, pp. 253–288. Edited by J. A. Bailey & J. W. Mansfield. Glasgow & London: Blackie.
- MAYAMA, S., MATSUURA, Y., IIDA, H. & TANI, T. (1982). The role of avenalumin in the resistance of oat to crown rust, *Puccinia coronata* f. sp. *avenae*. *Physiological Plant Pathology* **20**, 189–199.
- MOESTA, P. & GRISEBACH, H. (1982). L-2-Aminoxy-3-phenylpropionic acid inhibits phytoalexin accumulation in soybean with concomitant loss of resistance against *Phytophthora megasperma* f. sp. *glycinea*. *Physiological Plant Pathology* **21**, 65–70.
- NOBLE, J. P. & DRYSDALE, R. B. (1983). The role of benzoic acid and phenolic compounds in latency in fruits of two apple cultivars infected with *Pezizula malicorticis* or *Nectria galligena*. *Physiological Plant Pathology* **23**, 207–216.
- SWINBURNE, T. R. (1971). The infection of apples, cv. Bramley's Seedling, by *Nectria galligena* Bres. *Annals of Applied Biology* **68**, 253–262.
- SWINBURNE, T. R. (1975). Microbial proteases as elicitors of benzoic acid accumulation in apples. *Phytopathologische Zeitschrift* **82**, 152–162.
- WARD, E. W. B. (1983). Control mechanisms in phytoalexin production. In *Abstracts of the Proceedings of the 4th International Congress of Plant Pathology* (Melbourne, Australia, 17–25 August 1983), p. 61. Melbourne, Australia: Rowprint Services.
- WARTH, A. D. (1977). Mechanisms of resistance of *Saccharomyces baillii* to benzoic, sorbic and other weak acids used as food preservatives. *Journal of Applied Bacteriology* **43**, 215–230.
- YOSHIKAWA, M., YAMAUCHI, K. & MASAGO, H. (1978). Glyceollin: its role in restricting fungal growth in resistant soybean hypocotyls infected with *Phytophthora megasperma* var. *sojae*. *Physiological Plant Pathology* **12**, 73–82.