Neth. J. Pl. Path. 78 (1972) 179–185

In vitro and in vivo production of cell wall degrading enzymes by Botrytis cinerea from tomato

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Accepted 7 April 1972

Abstract

The production and activity of pectolytic and cellulolytic enzymes by *Botrytis cinerea* in tomato plants, as well as by conidia of this fungus in some nutrient media was studied. In inoculated tomato plants, the activity of these enzymes varied. PME, endo-PG and exo-PG were produced in petioles and fruits, while cellulase was only found in those parts which were softened by the invading mycelium. PGTE could only be detected in the softened parts of the petiole stumps. In suitable substrates, PME, endo-PG and exo-PG are produced rapidly with the onset of germination of the conidia. At some temperatures, endo-PG activity was found before germination could be observed. Some endo- and exo-PG was detected in washing water of conidia. The significance of the production of these enzymes by germinating conidia in relation to the infection process on tomato plants is discussed.

Introduction

When young unwounded tomato fruits are inoculated with conidia of *Botrytis cinerea* Pers. ex Fr., so-called *Botrytis*-'stips' or 'ghost spots' develop, due to penetration of germ tubes into epidermal cells. However, after inoculation of unwounded young or older tomato leaves, no penetration occurs (Verhoeff, 1970). There are at least three possible explanations for the differences between fruits and leaves as far as penetration by germ tubes of conidia of *B. cinerea* is concerned.

1. Fruits, but not leaves, produce exudates which stimulate germination of conidia and subsequent penetration, resulting in increased production of cell wall degrading enzymes (on fruits).
2. Fungitoxic compounds are exuded by leaves but not by fruits, or produced in greater quantity by leaves than by fruits.
3. Penetration is prevented by a berrier, which is present or is formed in leaves, but not in fruits.

Production of pectolytic and cellulolytic enzymes by *B. cinerea* was studied by Hancock et al. (1964). Using an isolate from onion, they found that the production of these enzymes was influenced by cultural conditions. *B. cinerea* produced pectin methylesterase (PME), cellulase (Cx), and an endo-polygalacturonase (endo-PG) in intact plants, in leaf sections and in potato dextrose broth. Exo-polygalacturonase

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(exo-PG) was found in inoculated leaf sections only, that is, galacturonic acid could be detected, in paper chromatography, after mixing extracts of the inoculated parts of the leaf with sodium polypectate. In their experiments no conidia were used for enzyme determinations. Barash (1968) reported the production of polygalacturonase by ungerminated spores of *Geotrichum candidum*. The amount of this enzyme increased rapidly during germination.

Before the epidermal cell wall proper can be reached, the cuticle has to be passed. Recently Shishiyama et al. (1970) described a cutin-esterase from *B. cinerea*, while the activity of this enzyme was measured on tomato cutin.

In this paper the activity of pectolytic and cellulolytic enzymes by *B. cinerea*, isolated from a tomato plant, was assayed in extracts of inoculated tomato tissues, as well as in filtrates of conidial suspensions, sampled at various time intervals after suspending.

Materials and methods

In all experiments with tomato plants, the variety 'Moneymaker' was used. *B. cinerea* was isolated from an infected tomato plant in a glasshouse; single spore isolates were subcultured on potato dextrose agar (P.D.A.) at room temperature. Inoculations were carried out either with actively growing mycelium on this medium or with spore suspensions, conidia being taken from 7 to 12 days old cultures on P.D.A., washed and resuspended in distilled water.

Inoculations were made by bringing a piece of agar with non-sporulating mycelium onto the freshly cut surface of a petiole stump, the petiole being cut at 5 cm from the stem. The inoculated plants, approximately 35 cm high, were kept at high r.h., at 23 °C, in a glasshouse. After 72 hours, inoculated petiole stumps were taken from the plants, washed in distilled water and cut into three pieces. Part 1 is the soft, rotten part of the stump, usually 1 cm from the site of inoculation. Part 2 is the next 0.5 cm from the inoculated stump; in this part hyphae are present but no external symptoms are visible. Part 3 is the healthy part of the stump. Pieces were ground in 0.1 *M* NaCl (1 g fresh weight per 10 ml salt solution); this mixture was than filtered through cheese cloth and subsequently centrifuged at 12000 *g* for 20 minutes. The supernatant was dialyzed against distilled water for 24 hours at 5 °C and stored at −20 °C until needed.

Young detached fruits, with a diameter of 2 to 3 cm, were wound-inoculated with a drop of spore suspension (15×10^3 conidia/ml). After 72 hours at 20 °C, the visibly infected parts were taken and extracted as described above. Non-detached fruits of the same size were also inoculated by dusting spores onto the surface. Inoculated fruits were wrapped in a polythene bag to maintain a high humidity. After 24 hours, they were sampled and peeled, the skin showing a great number of necrotic cells, as described earlier (Verhoeff, 1970). The skin was extracted as described above.

Enzyme activities of germinating conidia were assayed at different time intervals during the germination process at different temperatures in a nutrient medium or in distilled water, For enzyme determinations these media were centrifuged at 3000 *g* for 12 minutes, and the supernatants passed through a sterile millipore filter. Enzyme activity was assayed directly in the cell free filtrates after adding a suitable substrate to them. In these experiments, a concentration of 45×10^3 conidia/ml was used.

Pectin methylesterase (PME) activity was determined using a modification of the

continuous titration method as described by Hancock et al. (1964). To 30 ml of a 1.2% pectin solution (Fluka, A.G.) adjusted to pH 5.5, 5 ml of a test extract was added. Under continuous stirring at 30 °C, pH changes were checked every 15 minutes and readjusting the pH to the initial pH value was done with 0.02 N NaOH. With very active extracts, shorter time intervals were used. The quantity of NaOH used during 60 minutes was taken as a measure of enzyme activity which was expressed in μaeq/minute/ml extract. Heated extracts or non-inoculated tissues were used as controls.

Polygalacturonase activity was measured viscosimetrically (endo-PG) and using thin layer chromatography (exo-PG).

Endo-PG activity was determined according to the method described by Hancock et al. (1964). To 10 ml of a 1.2% sodium polypectate solution (Nutrit. Corp.), buffered at pH 4.5 with citric acid-phosphate buffer (McIlvaine), 5 ml of an extract was added, both substrate and extract being kept at 30 °C. Immediately after mixing, the mixture was put into a Ubbelohde 4 viscosity meter. Readings were taken every 3 to 5 minutes during the first 30 minutes of an experiment, and every 15 minutes thereafter. The activity is expressed as 1000/T50, T50 being the time in minutes required to reduce the viscosity of the mixture to 50%.

Exo-PG was estimated by a thin layer chromatographic assay (Ayers et al., 1969) of galacturonic acid and its oligo-uronides. To this end, extracts were added to a 1.2% sodium polypectate solution (1:1, v/v) buffered at pH 4.5; after various time intervals samples were assayed using silicagel F 254 (Merck) thin layer plates and ethylacetate-acetic acid-water (2:1:2) as the solvent system. After having been run, the plates were air-dried and re-run in the same direction using the same solvent. Spots were developed with a spray of either aniline phthalate (Merck) or aniline phosphate (2.5%) in acetic acid. After each spray, plates were heated at 95 °C for 5 minutes. Galacturonic acid and a mixture of its prepared oligo-uronides[2] were run as standards, all of which appeared as orange-brown spots.

Polygalacturonate transeliminase (PGTE) was measured by the viscosimetric method as described previously, the substrate in this case being buffered at pH 8.5, according to Chan and Sackston (1971). The thiobarbituric acid reaction, as described by Albersheim et al. (1960) was also used.

The activity of cellulase (Cx) was determined viscosimetrically as described by Hancock et al. (1964). The substrate used was 1.2% carboxymethyl cellulose (BDH Chemical Ltd) buffered at pH 5.5. The assay procedure used and the expression of enzymatic activity was the same as that described for endo-PG.

Results

The optimal pH for activity of PME, endo-PG and cellulase appeared to be 5.5, 4.5–5.0 and 5.5, respectively, with a rapid decrease in activity at lower and higher pH values.

The activities of cellulase and four pectolytic enzymes in extracts of inoculated tomato tissue are shown in Table 1. The figures represent the average of at least 5 replicates, after substraction of the figures obtained for heated extracts. No differences

[2] These were generously supplied by Mr. A. G. J. Voragen, Lab. for Food Technology, Agricultural University, Wageningen.

Table 1. Pectolytic and cellulolytic enzyme activity in various parts of a tomato plant, inoculated with *B. cinerea.*

	PME (μeq/min/ml)	endo-PG (1000/T50)	exo-PG	PGTE	Cellulase (1000/T50)
Petiole stumps:					
part 1 (softened by *B. cinerea*)	0.25	47	+[1]	+[1]	80
part 2 (no visible symptoms)	0.05	12	+	—	—
part 3 (healthy)	0.01	—	—	—	—
Fruits, wound inoculated	0.5	13	+[2]	—	40
Fruits-skin, 24 hours after inoculation	—	—	—	—	—

[1] + = present, — = absent
[2] Mono-, as well as di- and tri-galacturonic acid present.

Tabel 1. Activiteit van pectolytische en cellulolytische enzymen in verschillende delen van een tomateplant, geïnoculeerd met B. cinerea.

Table 2. Exo-polygalacturonase activity of germinating conidia of *B. cinerea* in a 1.2% sodium polypectate medium buffered at pH 5.4 in relation with temperature. Figures represent the average of the intensity of the colour of at least 5 spots on TLC plates, graded 1 ot 5; 1 approximately equivalent to 0.005 *M* galacturonic acid, 5 to 0.03 *M* of this compound.

Temperature in °C	Incubation time in hours													
	1		2		3		4		5		6		7	
	ga[1]	g[2]	ga	g	ga	g	ga	g	ga	g	ga	g	ga	g
5	—	—	—	—	—	—	—	—	—	—	—	—	—	—
15	—	—	—	+	—	+	—	+	—	+	—	+	0.5	34%
23	—	—	0.25	+	0.5	+	1	+	2	+	3	+	3.5	41%
26	—	—	—	+	—	+	0.25	+	0.5	+	2	+	2.5	24%
30	—	—	—	—	—	—	—	—	0.25	+	0.5	+	1.5	2%

[1] Colour intensity of the spots for mono galacturonic acid.
[2] Germination of the conidia; + = germination, — = no germination.

Tabel 2. Exo-polygalacturonase activiteit van kiemende conidiën van B. cinerea in een 1,2% natriumpolypectaatoplossing, gebufferd op pH 4,5, in samenhang met de temperattuur. De cijfers zijn het gemiddelde van de kleurintensiteit van 5 vlekken op kieselgel platen, uitgedrukt in de cijfers 1 tot 5; 1 is ongeveer gelijk aan 0,005 M galacturonzuur, 5 aan 0,03 M van deze stof.

in enzyme activity were found in the skin of inoculated and non-inoculated young fruits. PME was present in all extracts tested, endo-PG and exo-PG in all but one, viz. the healthy parts of inoculated petiole stumps. Cellulase appeared to be present in the softened part of inoculated petiole stumps and in the soft tissue of wound-inoculated fruits; PGTE was only present in the softened part of inoculated petiole stumps.

Parts 1 and 2 of inoculated petiole stumps showed distinct exo-PG activity, galacturonic acid becoming apparent within 10–20 minutes after incubation of the enzyme-substrate mixture. Upon incubation of extract-substrate mixtures of wound-inoculated

fruits di- and tri-galacturonic acid were revealed about 25 minutes after the extract had been mixed with the substrate.

In Table 2, results on exo-PG activity of germinating conidia are summarized. Galacturonic acid was found after 2 hours incubation at 23 °C, its amount increasing gradually. At lower and higher temperatures, the breakdown products of sodium polypectate were found later, viz. after 4 hours incubation at 26 °C, after 5 hours at 30 °C and after 7 hours at 15 °. No galacturonic acid was found within 7 hours at an incubation temperature of 5 °C. At this temperature, also no germination occurred within 7 hours. At 23 °C and 26 °C di- and tri-galacturonic acid were found after 5 hours incubation, at 30 °C di-galacturonic acid was found after 7 hours.

At 26 ° and 30 °C reduction in viscosity of the sodium polypectate substrate used as the germination medium began within 15 minutes, with a further decrease in viscosity during the following hours. At 23 °C a reduction in viscosity of the substrate occurred after 1 hour, while, at 20 °C, it took 2 hours. At 15 °C, a slight reduction in viscosity occurred after 6 hours, while at 5 °C none was found even after 7 hours. Fig. 1 shows the decrease in viscosity of 1.2% sodium polypectate at two temperatures.

In 1% glucose solution at 23 °C, germination can be seen after 1 hour. However, no endo-PG could be demonstrated within 16 hours incubation. When after this incubation time, the cell-free filtrate was concentrated ten times under vacuum, a slight reduction in viscosity of the substrate occurred, indicating a low endo-PG activity. In this mixture mono-, di-, and tri-galacturonic acid were found, in very weak spots on the TLC plates.

In similar experiments with conidia germinating in distilled water also some exo- and endo-PG activity could be found within 12 hours incubation at 23 °C; germina-

Fig. 1. Decrease in viscosity of a 1.2% sodium polypectate solution, buffered at pH 4.5, after mixing with conidia of *B, cinerea*. The viscosity of the mixture at zero time is 100%.

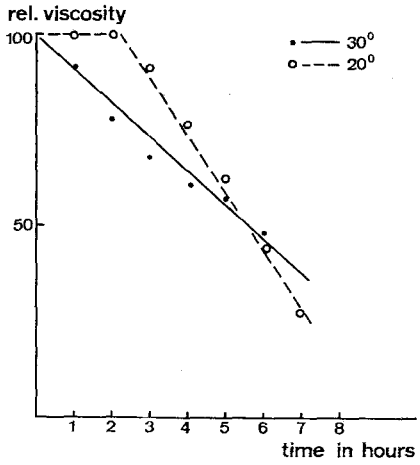

Fig. 1. Daling in viscositeit van een 1,2% natriumpolypectaatoplossing, gebufferd op pH 4,5 na mengen met een sporensuspensie van B. cinerea. De viscositeit van het mengsel onmiddellijk na het toevoegen van de sporensuspensie is op 100 gesteld.

tion was seen after 1 hour at this temperature. When conidia were washed in distilled water and this washing water was subsequently mixed with sodium polypectate, a slight reduction in viscosity was found; after 7 hours galacturonic acid could be detected.

When conidia were mixed with 1.2% pectin solution at 23 °C, PME activity was found after 2 hours of incubation, the activity increasing rapidly with longer incubation periods. Germination also started after 2 hours incubation in this medium.

No cellulase could be detected when conidia of *B. cinerea* were suspended in a 1.2% or 0.6% carboxymethyl-cellulose solution, buffered at pH 5.5. In this medium, hardly any germination was seen. When 1% glucose or 1% sacharose was used as the first substrate, also no cellulase activity was found within 24 hours at 23 °C. Germination of the conidia could be detected after about 1 hour in both media.

Discussion

As can be seen from Table 1, *B. cinerea* produces pectolytic and cellulolytic enzymes in inoculated tomato tissues. The amount of the various enzymes differed with mycelial development in the tissue, e.g. part 1 and part 2 of infected petiole stumps, and with the type of tomato tissue, viz. petioles or fruits. A similar effect of the substrate upon enzyme production was found by Hancock et al. (1964). From the polygalacturonases, endo-PG and exo-PG appeared to be present; PGTE was only found in the softened part of inoculated petioles.

The activity of the various enzymes produced by germinating conidia in different nutrient media also differed with the medium used, suggesting that these enzymes are inducible. However, in some cases endo-PG activity was found before germination was observed. This suggests that this enzyme is present in ungerminated conidia. The presence of some exo- and endo-PG in the washing water of conidia supports this. A similar result was obtained by Barash and Klein (1969) with conidia of *Geotrichum candidum*. In other cases, e.g. endo- and exo-PG activity in the sodium polypectate medium at 23 °C, enzyme activity was found at the time that germ tubes were observed, while at lower temperatures germination occurred without enzyme activity being detectable.

As could be expected, there is an influence of the temperature upon enzyme production. The optimum temperature for the enzymes tested was 30 °C, while germination of spores was optimal at about 23 °C. This might be the reason that a higher activity of exo- and endo-PG was found at 26 ° and 30 °C as compared with 15 °C, while germination was higher at this temperature (Table 2). The experiments described above clearly demonstrate that pectolytic enzymes are produced in a very early stage during the germination process when substrates are used which naturally occur in plants.

Shishiyama et al. (1970) showed cutinase activity with mycelium of *B. cinerea* from tomato on tomato cutin. There is no reason to doubt that such enzymes are produced by germ tubes. This implies that all enzymes necessary for penetration can be produced by *B. cinerea*, and other factors must be involved to explain the difference between fruit- and leaf-infection.

Samenvatting

In vitro en in vivo vorming van celwand-splitsende enzymen door Botrytis cinerea

In geïnoculeerde delen van een tomateplant blijkt de activiteit van deze enzymen afhankelijk te zijn van de aard van het aangetaste weefsel en de mate van aantasting (Tabel 1). Conidiën blijken in bepaalde media zeer snel pectolytische enzymen te vormen (Tabel 2, Fig. 1). Endo-PG activiteit kon bij 26° en 30 °C in een natriumpolypectaatmedium worden gemeten voordat kieming van de conidiën was opgetreden. Daar dit enzym, evenals het exo-PG, ook in waswater van conidiën kon worden aangetoond, zij het in zeer geringe hoeveelheid, lijkt aanwezigheid van deze twee enzymen in niet gekiemde conidiën aannemelijk. PME blijkt in een pectinebevattend medium te kunnen worden aangetoond op het moment, waarop kiembuizen zichtbaar worden. Cellulase kon daarentegen in een medium met kiemende sporen niet worden gevonden.

Acknowledgement

The technical assistance of miss J. I. Liem is gratefully acknowledged.

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