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Pectic enzymes associated with phosphate-stimulated infection of French bean leaves by Botrytis cinerea

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Abstract

Botrytis cinerea readily produced polygalacturonases and pectin esterases in shake cultures of Richards' medium containing orthophosphate and no pectinaceous material or galacturonic acid.

In inoculum droplets containing glucose and KH_2PO_4 or glucose and Na-ATP, which were used to evoke a susceptible reaction in French bean leaves, *B. cinerea* produced pectic enzymes. However, in inoculum droplets containing glucose but lacking phosphate, used to evoke a resistant reaction, activities of pectic enzymes remained low. As the enzyme activities already increased during the penetration stage of the infection process, it is assumed that these phosphate-stimulated activities of pectic enzymes are, at least partially, responsible for the phosphate-stimulated susceptible interaction between French bean leaves and *B. cinerea*.

Electrophoresis in pectin-polyacrylamide gels showed that two polygalacturonases with a high isoelectric point value were associated with the penetration stage of the infection process.

Additional keywords: polygalacturonases, pectin esterases, orthophosphate, ATP.

Introduction

Infection of intact leaves of French bean and other plant species by *Botrytis cinerea* Pers. : Fries is stimulated by glucose or other simple carbohydrates and inorganic or organic phosphate (Akutsu et al., 1981; Van den Heuvel, 1981). The carbohydrates promote superficial growth and formation of prepenetration structures, whereas phosphates stimulate penetration (Akutsu et al., 1981; Van den Heuvel and Waterreus, 1983).

The mechanism by which phosphate stimulates penetration is unknown. Phosphates are involved in many metabolic reactions in organisms. Stimulation of the activity of fungal polygalacturonases (Boothby, 1981) is a possible mechanism. Polygalacturonases and other pectic enzymes have been assigned an important role in the infection of plants by pathogenic fungi (e.g. Byrde, 1982; Cooper, 1983, 1984). However, although several reports have demonstrated the presence of pectic enzymes in *B. cinerea*-infected tissues, no clear picture of the involvement of these enzymes in tissue maceration and colonisation by this fungus has emerged (e.g. Verhoeff, 1980; Harris and Dennis, 1982; Brown and Adikaram, 1982, 1983; DiLenna and Fielding, 1983). Furthermore, so far no direct association of pectic enzyme activity with

penetration per se of *B. cinerea* into plant tissue has been reported. The only evidence for such an association comes from the swelling of the outer epidermal wall at the site at which the fungus penetrates the cuticle (McKeen, 1974; Mansfield and Richardson, 1981).

The aim of the present study was to find a possible correlation between pectic enzyme activity and phosphate-stimulated infection of French bean leaves by *B. cinerea,* and to indicate which pectic enzymes, if any, are associated in particular with penetration.

Materials and methods

Plants and fungus. French bean plants *(Phaseolus vulgaris* L. cv. Dubbele Witte zonder draad) and sporulating cultures of isolate BC-1 of *B. cinerea* were grown as described previously (Van den Heuvel and Waterreus, 1983).

Shake cultures of the fungus were grown as follows. Erlenmeyer flasks with (slightly modified) Richards' solution were inoculated with a washed conidial suspension of *B. cinerea* (final concentration 2×10^4 conidia ml^{-1}). The medium contained 0.11 M glucose, 99 mM KNO_3, 37 mM KH_2PO_4, 1 mM $MgSO_4.7H_2O$ and 2.5 μM $FeCl_3$ in 0.1 M 2(N-morpholino)ethanesulfonic acid (MES) buffer (pH 5.0). In some experiments a Richards' medium was used from which the KH_2PO_4 had been omitted. The flasks were incubated at 19 to 20 °C on a reciprocal shaker (c. 70 strokes min^{-1}) for up to 7 days. After incubation the mycelium was separated from the culture liquid by filtration over Whatman nr. 4 filter paper. For determination of dry mycelial weight preweighed filter papers with mycelium were dried at 80 °C for 24 h and weighed. Culture filtrates were dialysed against 0.01 or 0.02 M phosphate buffer (pH 5.0) for 24 h and kept at below −20 °C until use.

Inoculation. The preparation of conidial suspensions, the inoculation of detached leaves with 5-μl droplets of suspension (2×10^6 conidia ml^{-1}) and conditions of incubation of inoculated leaves were as described earlier (Van den Heuvel, 1981). Composition of inocula varied with the experiments. Five or six leaves were inoculated (40 droplets per leaf) per replicate treatment. In general, duplicate or triplicate treatments were applied per experiment.

After various incubation times at 19 °C the inoculum droplets were rinsed from the leaves by spraying the leaves twice with a small aliquot of 0.02 M phosphate buffer solution (pH 5.0) (in total 0.6 or 1.0 per leaf). The leaf washings were combined per treatment. In general, particles were removed from the leaf washings by centrifugation (1400 *g,* 10 min) or filtration (membrane filter with 1.2 μm pores). For most enzyme assays the leaf washings were dialysed overnight against 0.02 M phosphate buffer (pH 5.0) at 2 °C, and stored at below −20 °C until use.

Protein assay. Protein concentrations were determined with the method of Bradford (1976), with bovine serum albumin as the standard.

Enzyme assay methods. The pectin and sodium polygalacturonate used as substrates were from Fluka AG (nr. 76280) and Sigma Chem. Co. (nr. P-1879), respectively.

Pectin esterases. Pectin esterase (PE) activity was determined at pH 5.5 by the

titration method described by Verhoeff and Warren (1972). Enzyme activity was expressed as μeq NaOH added min^{-1} ml^{-1} culture filtrate or inoculum.

Polygalacturonases. Polygalacturonase (PG) activities were determined by three different methods.

(a) Measurement of the increase in reducing groups released from sodium polygalacturonate in reaction mixtures according to the method of Nelson (1944) as modified by Somogyi (1952). Reaction mixtures contained 1.0 ml of enzyme solution and 6.5 or 9.0 ml of 0.1% sodium polygalacturonate in 0.1 M acetate buffer (pH 5.0). At regular intervals after incubation at 30 °C 1.0-ml samples were taken for determination of reducing groups, with galacturonic acid as the standard. Activity was defined as μmoles reducing groups released h^{-1} ml^{-1} culture filtrate or inoculum.

(b). Viscosimetric assay. Reaction mixtures contained 1.0 or 2.0 ml of an enzyme solution and 14.0 or 13.0 ml, respectively, of a solution containing either 1.0% pectin in 0.1 M acetate buffer (pH 5.0) or 2.4% sodium polygalacturonate and 0.07 M sodium oxalate in 0.1 M acetate buffer (pH 5.0), unless mentioned otherwise. The sodium oxalate was added to enhance the solubility of sodium polygalacturonate. At intervals the viscosity of the reaction mixtures, which were held at 30 °C, was measured with an Ubbelohde nr. 4 viscosimeter. Enzyme activity was expressed normally as $1000/T_{50}$ ml^{-1} culture filtrate or inoculum, T_{50} being the time (min) required to reduce the viscosity to 50%. In case of very low activities, however, enzyme activity was expressed as % reduction of viscosity h^{-1} ml^{-1} culture filtrate or inoculum.

(c) Cup plate assay. Cup plate assays were a slight modification of the method described by Dingle et al. (1953). Aliquots of 150 μl enzyme solution were assayed in 9.0-mm-wide wells (cups) punched in 3.3-mm-thick layers of agar adjusted to pH 5.0. Enzyme activity was expressed as area (mm^2) of clear zone around a cup.

Pectin and pectate lyase. Selected enzyme solutions were assayed qualitatively for pectin and pectate lyase activity with the thiobarbituric acid reaction described by Ayers et al. (1966).

Pectin-polyacrylamide gel electrophoresis of pectic enzymes. Electrophoresis of pectic enzymes in polyacrylamide gels amended with pectin was based upon the method described by Cruickshank and Wade (1980) and Cruickshank (1983), but strongly modified.

Two-mm-thick 11.5 × 25 cm gels were prepared using a pectin-acrylamide mixture of the following composition: in 100 ml 0.05 M Tris-0.1 M glycine buffer (pH 8.7) were dissolved 7.58 g acrylamide, 0.205 g N,N′-methylenebisacrylamide, 0.154 ml N,N,N′,N′-tetramethylethylenediamine, 0.092 g pectin, 16.5 mg sodium azide and, just prior to pouring, 73.8 mg ammonium persulfate.

For electrophoresis the LKB 2117 Multiphor system was used. The electrode tanks contained the same Tris-glycine buffer (pH 8.7) as used in the gel. After pre-electrophoresis of the gel for 30 min, the wells were filled with 5-μl enzyme samples and 1-μl spots of 0.05% bromophenol blue in gel buffer were applied to the cathodic side of the sample wells. Electrophoresis was subsequently carried out at 10 °C and at constant power of 25 W for 2 h.

Detection of pectic enzymes by incubation of the gel in 0.1 M DL-malic acid and staining of the gel with ruthenium red was according to the method of Cruickshank and Wade (1980) as modified by Cruickshank (1983).

Results

Enzyme production in shake culture. In shake cultures of Richards' medium (without a pectinaceous substrate) *B. cinerea* readily produced pectic enzymes. In filtrates of 6- or 7-day-old cultures considerable PG and PE activities were detected (Table 1). No evidence was found for production of pectin or pectate lyase, as determined with the thiobarbituric acid reaction, using either pectin or sodium polygalacturonate as substrate, each buffered at pH 5.0 or 9.3, and in the presence or absence of 1 mM $CaCl_2$. These two pH values were selected as reduction of viscosity of sodium polygalacturonate by the culture filtrates at 30 °C was maximal at pH 4.5 to 5.0 and 9.0 to 9.5 (Fig. 1). As no pectin or pectate lyase was detected, reduction of viscosity of pectin or sodium polygalacturonate may be ascribed to PG activity solely.

Table 1. Activities of pectic enzymes in filtrates of 6- or 7-day-old cultures of *B. cinerea* in Richards' medium.

Enzyme	Assay method/substrate	Activity
Polygalacturonase[1]	Viscosimetry/sodium polygalacturonate	$1000/T_{50}$ (min) ml^{-1} = 53
Polygalacturonase	Viscosimetry/pectin	$1000/T_{50}$ (min) ml^{-1} = 286
Polygalacturonase	Reducing group assay/ sodium polygalacturonate	61.2 μmoles reducing groups h^{-1} ml^{-1}
Pectin esterase	Titration/pectin	0.231 μeq NaOH min^{-1} ml^{-1}

[1] Qualitative tests for pectin or pectate lyase were negative (see text).

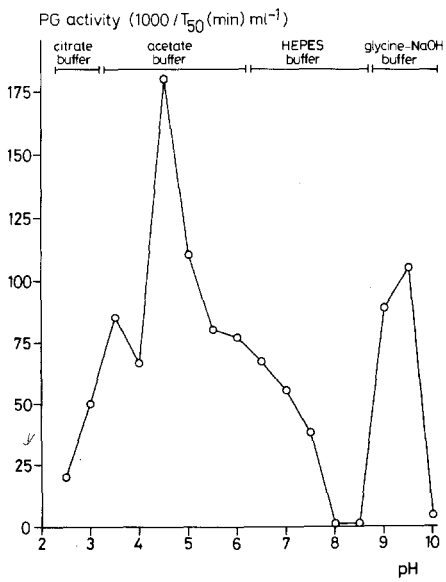

Fig. 1. PG activity (measured viscosimetrically with sodium polygalacturonate as substrate) at various pH's of a dialysed filtrate of a 6-day-old *B. cinerea* shake culture grown in Richards' medium. Qualitative tests for pectin or pectate lyase were negative (see text). pH's < 3.5 and > 6.0 of the reaction mixtures were obtained by replacing the acetate buffer by citrate, 2-[4-(2-hydroxyethyl)-1-piperazinyl]ethanesulfonic acid (HEPES) or glycine-NaOH buffer.

Stimulation of PG activity in culture by phosphate. Much more PG activity was found in filtrates of *B. cinerea* cultures grown for four or more days on 'normal' Richards' medium than in similar culture filtrates from Richards' medium from which KH_2PO_4 had been omitted (Fig. 2). This was true not only when PG activity was expressed per ml medium, but also when activity was expressed per dry weight of mycelium, except for cultures in phosphate-free medium whose high values during the lag phase were due to a low mycelial weight.

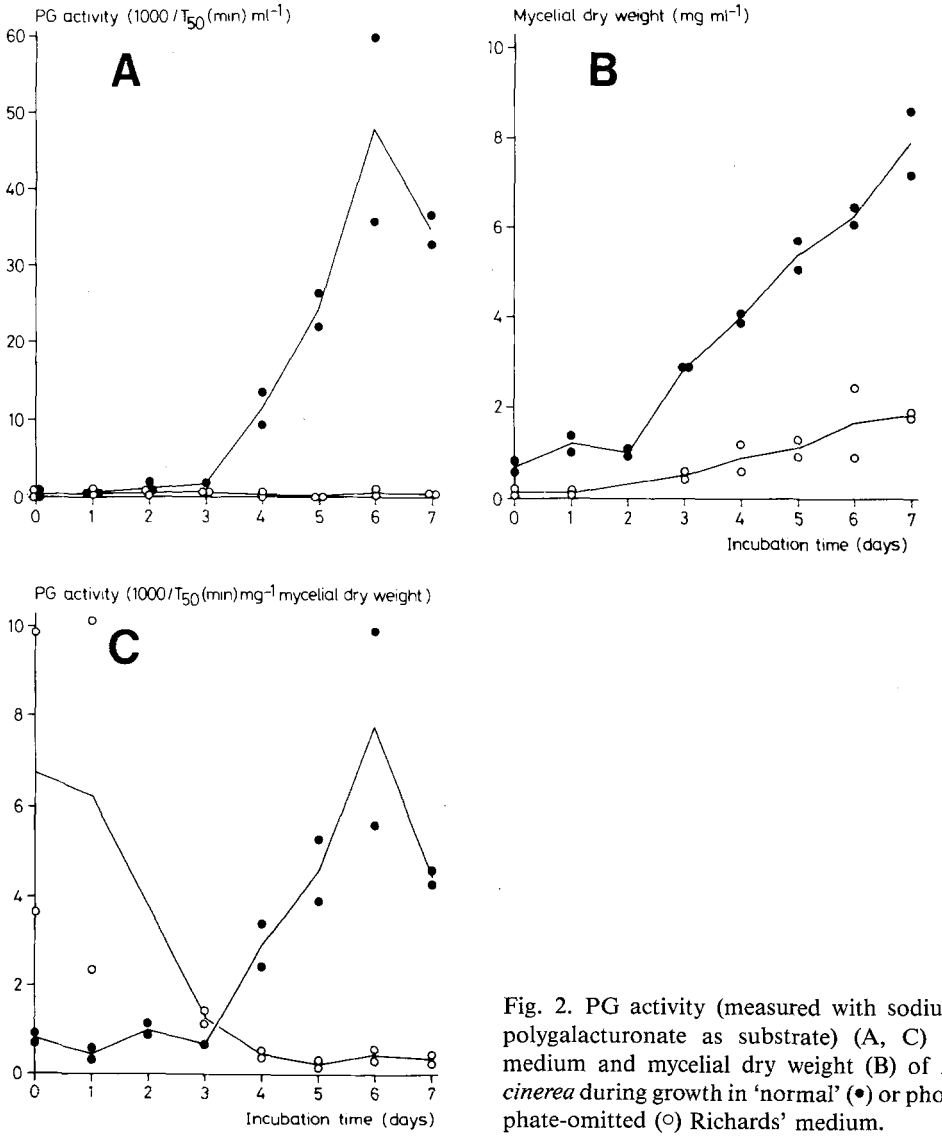

Fig. 2. PG activity (measured with sodium polygalacturonate as substrate) (A, C) in medium and mycelial dry weight (B) of *B. cinerea* during growth in 'normal' (•) or phosphate-omitted (○) Richards' medium.

Activities of pectic enzymes in inoculum droplets. Infection of bean leaves by *B. cinerea* is dependent on the composition of inoculum droplets (Van den Heuvel and Waterreus, 1983). Five-μl droplets containing 2×10^6 conidia ml^{-1} of 0.11 M glucose + either 67 mM KH_2PO_4 or 1 mM Na-ATP give rise to spreading lesions (susceptible reaction), whereas similar inoculum droplets in which the conidia are suspended in 0.11 M glucose without phosphate or ATP normally give rise to a superficial browning only (resistant reaction) in the tissue covered by the inoculum droplets.

In inoculum droplets giving rise to a susceptible reaction pectic enzymes were readily detected (Fig. 3). Although considerable variations were observed in some cases, the enzyme activities increased steadily with increasing incubation time. The variations were most pronounced at more than 24 h after inoculation, when inoculum droplets could have been taken up partially by the lesion tissues; but differing fungal development rates may also have accounted for varying enzyme activities. In inoculum droplets giving rise to a resistant reaction, only very low activities of pectic enzymes were found. No pectate lyase and only a trace of pectin lyase were detected in inoculum droplets above spreading lesions, collected at 48 h after inoculation with 2×10^6 conidia ml^{-1} 0.11 M glucose + 67 mM KH_2PO_4. Therefore, enzyme activities measured by viscosimetric, reducing group or cup plate assays must have been due almost exclusively to PG's. PG activities measured viscosimetrically at pH 9.3 were in general lower than those measured at pH 5.0, but relative changes in activity with time were similar.

Control droplets, without conidia and containing 0.11 M glucose + 67 mM KH_2PO_4, were incubated on bean leaves at 19 °C for 44 h. As measured viscosimetrically at pH 5.0 with pectin or sodium polygalacturonate as substrate, only a trace of PG was detected, whereas no PG activity was found with the reducing group assay. PE or pectin or pectate lyase were not detected either.

In order to assess a possible involvement of pectic enzymes in the penetration of bean leaves by *B. cinerea* the activity of pectic enzymes in infection droplets was analysed in more detail. Numbers of penetrations were determined light-microscopically by the procedure described by Van den Heuvel and Waterreus (1983). Notwithstanding a considerable variation in results, Fig. 4 shows a clear correlation in time between the number of penetrations in French bean leaves and the appearance of PG activity measured in different ways. In inoculum droplets containing glucose and KH_2PO_4 penetration was detected from 8 h after inoculation onwards. It was associated with an increasing PG activity; during this period only traces of PE were detected. In inoculum droplets containing glucose but no phosphate, no penetration was observed, while PG activities remained negligible and, in most cases, far below those determined in inoculum droplets with glucose and phosphate.

Electrophoresis of pectic enzymes. Electrophoresis of dialysed filtrates of 6-day-old *B. cinerea* shake cultures grown in phosphate-containing Richards' medium revealed at least five white (PG) bands and two dark red (PE) bands (Fig. 5). Under conditions suitable for detecting pectin or pectate lyase activity (Cruickshank and Wade, 1980; Cruickshank, 1983) orange, yellow or additional white bands, which are characteristic for these enzymes, were not observed. The bands were tentatively labeled PG1 to 5 and PE1 and 2 (Fig. 5). Apparently PG2, PG3 and PE1 were the most prominent pectic enzymes in the culture filtrates.

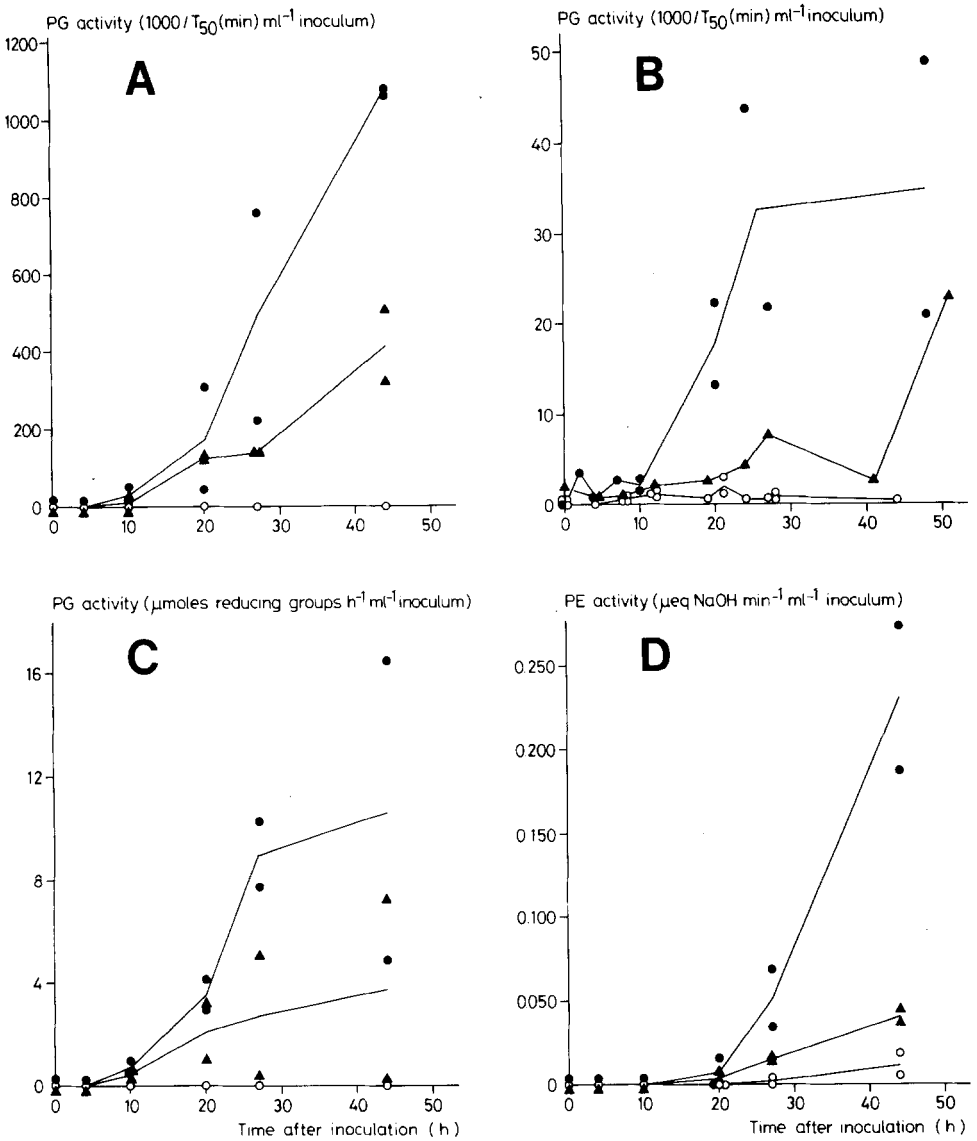

Fig. 3. PG activity (measured either viscosimetrically with pectin (A) or sodium polygalacturonate (B) as substrate or using the reducing groups assay (C)) and PE activity (D) in inoculum droplets on French bean leaves at various times after inoculation. Inocula consisted of conidia suspended in 0.11 M glucose + 67 mM KH_2PO_4 (•), in 0.11 M glucose + 1 mM Na-ATP (▲) or in 0.11 M glucose (○).

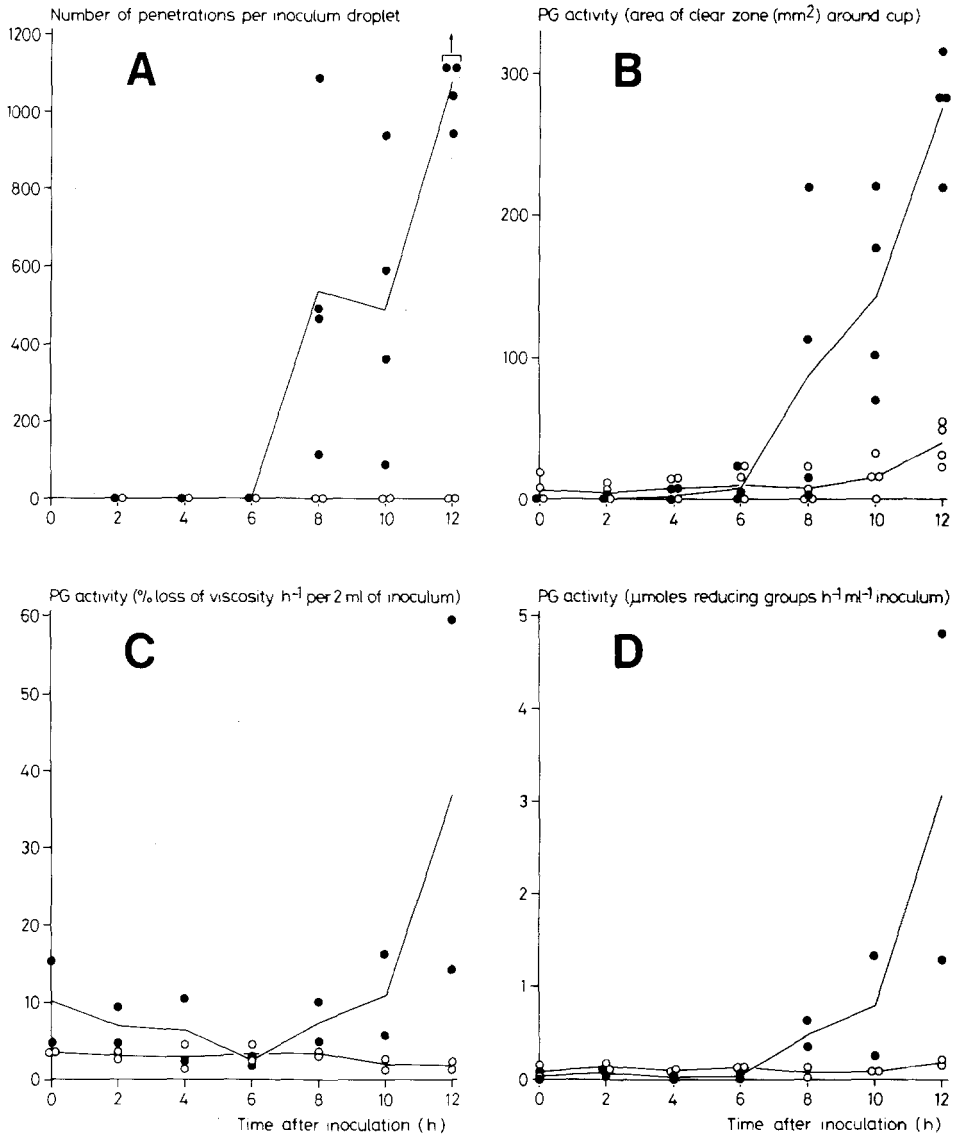

Fig. 4. Number of penetrations of *B. cinerea* in French bean leaves (A) and PG activity (measured with sodium polygalacturonate as substrate by cup plate assay (B), viscosimetry (C) or reducing groups assay (D)) in inoculum droplets on leaves at various times after inoculation. Inocula consisted of conidia suspended in 0.11 M glucose + 67 mM KH_2PO_4 (•) or in 0.11 M glucose (○).

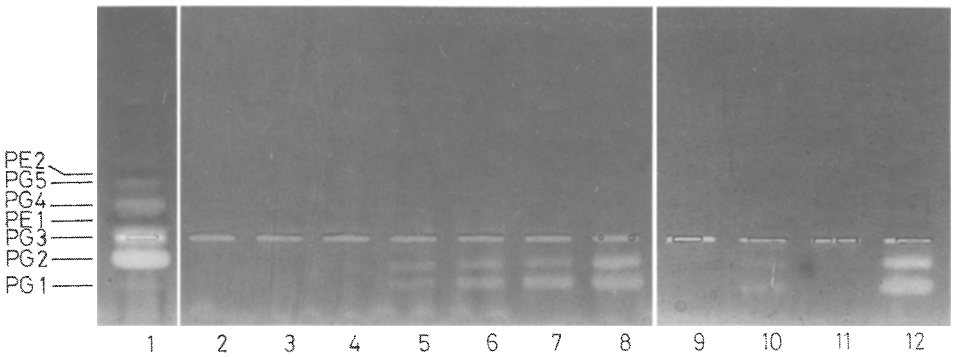

Fig. 5. Pectin-polyacrylamide gel electrophoresis of pectic enzymes of *B. cinerea*. PG1 to PG5 : polygalacturonases; PE1 and PE2: pectin esterases. Lane 1: filtrate of 6-day-old shake culture in Richards' medium; lanes 2 to 8: inoculum droplets (2×10^6 conidia ml^{-1} 0.11 M glucose + 67 mM KH_2PO_4) collected from leaves 0, 2, 4, 6, 8, 10 and 12 h, respectively, after inoculation; lanes 9 to 12: inoculum droplets (2×10^6 conidia ml^{-1} distilled water, 67 mM KH_2PO_4, 0.11 M glucose, and 0.11 M glucose + 67 mM KH_2PO_4, respectively) collected from leaves 10 h after inoculation.

The location of the bands near the slots indicates that all enzymes must have a fairly high isoelectric point (pI) value, i.e. around 9. Occasionally, a few very small white or red bands were detected more closely to the anode.

Inoculum droplets containing 2×10^6 conidia of *B. cinerea* ml^{-1} 0.11 M glucose + 67 mM KH_2PO_4 were collected from leaves with a Pasteur pipette up to 12 h after inoculation. The suspensions were centrifuged (1400 *g*, 10 min) and the supernatants were used for gel electrophoresis. Fig. 5 shows the appearance of exclusively PG1 and PG2 in the inoculum droplets. Occasionally also traces of PE1 were detected at 10 to 12 h after inoculation.

PG1 and PG2 also appeared, albeit at a low activity, in inoculum droplets in which conidia had been suspended in 67 mM KH_2PO_4, but not in those in which conidia had been suspended in distilled water or in 0.11 M glucose (Fig. 5).

No pectic enzymes were detected in droplets of conidial suspensions containing glucose with or without added KH_2PO_4, which had been placed on glass slides and incubated for up to 12 h under the same conditions as used with leaves.

Discussion

B. cinerea readily produced several PG's and PE's in Richards' medium not containing a pectinaceous substrate. This confirms earlier reports (Tani and Nanba, 1969; Szajer and Bousquet, 1975; Martinez et al., 1982) that many, if not all, pectic enzymes produced by this pathogen appear to be constitutive. However, comparison with a medium from which phosphate was omitted, indicates that phosphate plays a stimulating role in the activity of PG's. Whether this stimulation by phosphate is a stimulation of the synthesis or release of the enzymes, an enhancement of their activity or a protection against inactivation, as was suggested for PG of *Phialophora radicicola* var. *radicicola* (Boothby, 1981), will be the object of further research.

The presence of a phosphate in addition to a simple carbohydrate in inoculum droplets of *B. cinerea* had already been shown to be necessary for a susceptible reaction (Akutsu et al., 1981; Van den Heuvel, 1981). This stimulation by phosphate now appeared to be associated with a stimulation of the activity of pectic enzymes. This may be concluded from the experiments in which the pectic enzyme activities in inoculum droplets containing KH_2PO_4 or Na-ATP always increased, whereas those in inoculum droplets without any phosphate always remained negligible. As enzymes present in infected tissue may diffuse into the inoculum droplets, the activities of these enzymes in the inoculum droplets will reflect their activities in the underlying infected tissue.

Differences in enzyme activity between KH_2PO_4- and Na-ATP-amended inoculum droplets parallelled differences in the rate of fungal penetrations (Van den Heuvel and Waterreus, 1983; unpublished results). In susceptible reactions starting from KH_2PO_4-amended inoculum droplets, most penetrations occurred between 6 and 12 h after inoculation and in those from Na-ATP-amended inocula between 12 and 24 h after inoculation.

Activity of pectic enzymes appeared already to be enhanced during the penetration stage of a susceptible infection, whereas in a resistant interaction such an enhancement did not occur. The enhancement of pectic enzyme activity started at or slightly before the appearance of infection hyphae, but probably after penetration of the cuticle. This indicates that pectic enzymes are involved in the penetration of the outer epidermal cell wall, although a primary role for these enzymes in the penetration cannot yet be established. This role will be investigated in further studies.

The large variations in PG activities observed during the penetration stage of the infection process may have been due primarily to the large variations in the penetration rate of *B. cinerea*. Penetrations and further fungal development were, in general, rather uniform per leaf. Variations were largest between leaves. So far, no leaf characteristics have been found that are associated with the differences in infection rate.

In conclusion, this report presents evidence in favour of the hypothesis that the stimulation by phosphate of the infection of French bean leaves by *B. cinerea* is due to a stimulation of the penetration through the enhancement of the activity of certain pectic enzymes by phosphates. The principal enzymes involved in penetration seem to be two PG's with a high isoelectric point value. These enzymes were also associated, together with other PG's as well as PE's, with fungal colonisation of the leaf tissues.

Acknowledgements

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Samenvatting

Vorming van pectolytische enzymen tijdens de door fosfaat gestimuleerde infectie van bonebladeren door Botrytis cinerea

Botrytis cinerea vormde vlot polygalacturonasen en pectineësterasen in schudculturen

in Richards' medium dat wel orthofosfaat maar geen pectineachtig materiaal of galacturonzuur bevatte.

In inoculumdruppels met glucose + KH_2PO_4 of met glucose + Na-ATP, waarmee een vatbare reactie in bonebladeren kon worden opgewekt, vormde *B. cinerea* verschillende pectolytische enzymen. In inoculumdruppels met glucose maar zonder fosfaat, waarmee een resistente reactie werd teweeggebracht, bleef de activiteit van pectolytische enzymen gering.

Aangezien de enzymactiviteiten al begonnen toe te nemen tijdens de binnendringingsfase van het infectieproces, wordt verondersteld dat deze door fosfaat gestimuleerde activiteiten van pectolytische enzymen althans ten dele verantwoordelijk zijn voor de door fosfaat gestimuleerde vatbare interactie tussen bonebladeren en *B. cinerea.*

Elektroforese in pectine-polyacrylamidegels liet de toename zien van twee polygalacturonasen met een hoog isoëlektrisch punt tijdens de binnendringingsfase van het infectieproces.

References

Akutsu, K., Kobayashi, Y., Matsuzawa, Y., Watanabe, T., Kō, K. & Misato, T., 1981. Morphological studies on infection process of cucumber leaves by conidia of *B. cinerea* stimulated with various purine-related compounds. Ann. phytopath. Soc. Japan 47: 234-243.

Ayers, W.A., Papavizas, G.C. & Diem, A.F., 1966. Polygalacturonate trans-eliminase and polygalacturonase production by *Rhizoctonia solani.* Phytopathology 56: 1006-1011.

Boothby, D., 1981. Enhancement of polygalacturonase activity by potassium phosphate in liquid culture of *Phialophora radicicola* var. *radicicola.* Trans. Br. mycol. Soc. 77: 639-641.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analyt. Biochem. 72: 248-254.

Brown, A.E. & Adikaram, N.K.B., 1982. The differential inhibition of pectic enzymes from *Glomerella cingulata* and *Botrytis cinerea* by a cell wall protein from *Capsicum annuum* fruit. Phytopath. Z. 105: 27-38.

Brown, A.E. & Adikaram, N.K.B., 1983. A role for pectinase and protease inhibitors in fungal rot development in tomato fruits. Phytopath. Z. 106: 239-251.

Byrde, R.J.W., 1982. Fungal 'pectinases': from ribosome to plant cell wall. Trans. Br. mycol. Soc. 79: 1-14.

Cooper, R.M., 1983. The mechanisms and significance of enzymic degradation of host cell walls by parasites. In: Callow, J.A. (Ed.), Biochemical plant pathology. John Wiley & Sons Ltd, New Yord, p. 101-135.

Cooper, R.M., 1984. The role of cell wall-degrading enzymes in infection and damage. In: Wood, R.K.S. & Jellis, G.J. (Eds), Plant diseases: infection, damage and loss. Blackwell Sci. Publ., Oxford, London, Edinburgh, Boston, Palo Alto, Carlton, p. 13-27.

Cruickshank, R.H., 1983. Distinction between *Sclerotinia* species by their pectic zymograms. Trans. Br. mycol. Soc. 80: 117-119.

Cruickshank, R.H. & Wade, G.C., 1980. Detection of pectic enzymes in pectin-acrylamide gels. Analyt. Biochem. 107: 177-181.

DiLenna, P. & Fielding A.H., 1983. Multiple forms of polygalacturonase in apple and carrot tissue infected by isolates of *Botrytis cinerea.* J. gen. Microbiol. 129: 3015-3018.

Dingle, J., Reid, W.W. & Solomons, G.L., 1953. The enzymic degradation of pectin and other polysaccharides. II. Application of the 'cup-plate' assay to the estimation of enzymes. J. Sci. Fd Agric. 4: 149-155.

Harris, J.E. & Dennis, C., 1982. The influence of berries infected with *Botrytis cinerea* on the enzymic breakdown of sulphited strawberries. Ann. appl. Biol. 101: 109-117.
Heuvel, J. van den, 1981. Effect of inoculum composition on infection of French bean leaves by conidia of *Botrytis cinerea.* Neth. J. Pl. Path. 87: 55-64.
Heuvel, J. van den & Waterreus, L.P., 1983. Conidial concentration as an important factor determining the type of prepenetration structures formed by *Botrytis cinerea* on leaves of French bean *(Phaseolus vulgaris).* Pl. Path. 32: 263-272.
Mansfield, J.W. & Richardson, A., 1981. The ultrastructure of interactions between *Botrytis* species and broad bean leaves. Physiol. Pl. Path. 19: 41-48.
Martinez, M.J., Reyes, F. & Lahoz, R., 1982. Cell wall plant polysaccharide-degrading enzymes in autolysis of *Botrytis cinerea.* Trans. Br. mycol. Soc. 78: 395-403.
McKeen, W.E., 1974. Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea.* Phytopathology 64: 461-467.
Nelson, N., 1944. A photometric adaptation of the Somogyi method for the determination of glucose. J. biol. Chem. 153: 375-380.
Somogyi, M., 1952. Notes on sugar determination. J. biol. Chem. 195: 19-23.
Szajer, I. & Bousquet, J.F., 1975. Production en culture d'endopolygalacturonase et de pectineméthylestérase par des champignons phytopathogènes: quelques aspects de l'influence de la source de carbone du milieu nutritif. Ann. Phytopath. 7: 299-307.
Tani, T. & Nanba, H., 1969. Qualitative nature of macerating activities in the culture filtrates of *Botrytis cinerea.* Ann. phytopath. Soc. Japan 35: 1-9.
Verhoeff, K., 1980. The infection process and host-pathogen interactions. In: Coley-Smith, J.R., Verhoeff, K. & Jarvis, W.R. (Eds), The biology of *Botrytis.* Academic Press, London, New York, Toronto, Sydney, San Francisco, p. 153-180.
Verhoeff, K. & Warren, J.M., 1972. In vitro and in vivo production of cell wall degrading enzymes by *Botrytis cinerea* from tomato. Neth. J. Pl. Path. 78: 179-185.