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Sequential production of polygalacturonase, cellulase, and pectin lyase by *Rhizoctonia solani*[1]

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The sequence of appearance of cell wall degrading enzymes of *Rhizoctonia solani* propagules was followed. Polygalacturonase (PG; EC 3.2.1.15) was induced earlier by sodium polypectate (NaPP) as compared with the induction of cellulase (Cx; EC 3.2.1.4) by carboxymethyl cellulose (CMC), cellobiose, or fibrous cellulose powder. Increasing CMC concentration to 0.5% shortened the time of Cx appearance. In Czapek medium containing citrus pectin, pectin lyase (PL; EC 4.2.2.10) was produced faster and at higher amounts than in a medium containing NaPP as the sole carbon source. PG appearance also preceded that of PL in media simultaneously supplemented with their respective inducers. NaPP, which induced production of PG, repressed Cx production. Among the Cx inducers, only CMC and cellobiose repressed PG production to any extent. At pH 6.0, either in a synthetic medium or on autoclaved bean hypocotyl segments, a delay in PG production as compared with Cx and PL production was observed. Optimal pH levels for enzyme production and activity were 4.0 and 5.0 for PG, 4.0 and 5.5 for Cx, and 8.0 and 7.5 for PL. PG was less repressed than Cx by glucose, cellobiose, and monogalacturonic acid, while PL was not affected.

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Nous avons suivi la séquence d'apparition des enzymes dégradant la paroi cellulaire chez des propagules de *Rhizoctonia solani*. La polygalacturonase (PG; EC 3.2.1.15) est induite en premier lieu par le polypectate de sodium (NaPP) en comparaison de l'induction de la cellulase (Cx; EC 3.2.1.4) par la carboxyméthyl cellulose (CMC), la cellobiose ou la cellulose fibreuse en poudre. L'accroissement de la concentration de CMC jusqu'à 0.5% raccourcit le temps d'apparition de la Cx. Dans un milieu Czapek contenant de la pectine de citrus, la lyase pectine (PL; EC 4.2.2.10) est produite plus rapidement et à de plus grandes quantités que dans un milieu contenant le NaPP comme seule source de carbone. L'apparition de la PG précède aussi celle de la PL dans des milieux simultanément enrichis avec leur inducteur respectif. Le NaPP qui induit la production de la PG, réprime aussi la production de la Cx. Parmi les inducteurs de la Cx, seulement la CMC et la cellobiose répressent la production de la PG peu importe la concentration. A un pH 6.0, dans un milieu synthétique ou dans des segments autoclavés d'hypocotyl de la fève, nous observons un délai dans la production de la PG en comparaison de celles de la Cx et de la PL. Les niveaux optimum de pH pour la production et l'activité enzymatique sont 4.0 et 5.0 pour la PG, 4.0 et 5.5 pour la Cx et 8.0 et 7.5 pour la PL. La PG est moins réprimée que la Cx par le glucose, la cellobiose et l'acide monogalacturonique, alors que la PL n'est pas affectée. [Traduit par le journal]

Introduction

Cell wall degrading enzymes are produced by many plant pathogens. Polygalacturonase (PG; poly(1,4-α-D-galacturonide) glycanohydrolase, EC 3.2.1.15) and pectin lyase (PL; poly(methoxygalacturonide)lyase, EC 4.2.2.10) cause plant cell maceration and death of plant tissues (6,14,18, 19), while cellulase (Cx; 1,4-(1,3;1,4)-β-D-glucan 4-glucanohydrolase, EC 3.2.1.4) assists pathogen penetration into the host cell (5). In *Colletotrichum lindemuthianum* (8), *Pyrenochaeta terrestris* (10), and *Fusarium oxysporum* f. sp. *lycopersici* PG is detected before Cx (12). During the initial stages of pathogenesis in bean seedling tissue infected with *Rhizoctonia solani*, a high rate of production of endo-PG as compared with Cx was noted (23). It has been recently shown that PG appearance in *R. solani* propagules is concomitant with germination, precedes the appearance of Cx (16), and is more easily released from cells than Cx (15). In autoclaved bean segments inoculated with *R. solani*, PG and PL appear simultaneously by the 6th day; PG disappears after 18 days when the pH of the medium rises from 6 to 8 (6).

A pronounced repression in PG, PL, and Cx

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production was observed when glucose or monogalacturonic acid was added to the inoculated growth medium (7, 9, 10, 20, 22, 24). Thus, in *Pyrenochaeta terrestris*, Horton and Keen (10) attributed the late appearance of Cx as compared with PG to a greater glucose catabolic repression of Cx.

This work describes the sequential appearance of PG, PL, and Cx in media inoculated with *R. solani* and the factors affecting this sequence.

Materials and Methods

Preparation of Propagules

Rhizoctonia solani Kühn propagules were prepared by blending mats of mycelium and washing in buffer as described elsewhere (16). Only the large propagule fractions (250–500 μm) were used. Samples equivalent to 5 mg dry weight (80C) were used for inoculation, unless otherwise stated.

Enzyme Production and Preparation of Growth Media

Growth and enzyme production were followed in 250-ml Erlenmeyer flasks containing 13 ml growth medium, were inoculated, and then were incubated in a shaker water bath at 27C, unless otherwise stated. To induce enzyme production, the propagules were inoculated in a modified Czapek medium in which the sole carbon source was either sodium polypectate (NaPP, Sigma Chemical Co.) for PG, citrus pectin grade I (Sigma) for PL, or carboxymethyl cellulose (CMC, BDH), Whatman fibrous cellulose powder (CF 1), or β-D-cellobiose (Sigma) for Cx. The pH was adjusted to 4.0 for PG and Cx and to 8.0 for PL unless otherwise stated. In all cases, the media were autoclaved at low pH (5.0–5.5) and later adjusted to the desired pH by using 0.1 M HCl or 0.1 M NaOH. In certain experiments, citrate, phosphate, and tris(hydroxymethyl)aminomethane (Tris-HCl, Tham, Fisher Scientific Co.) buffers were used to adjust the pH at ranges between 3.5 and 6.0, 6.0 and 7.5, and 7.5 and 9.0, respectively. When glucose, cellobiose, or α-D-monogalacturonic acid (MGA, Sigma) solutions were used, they were autoclaved separately before mixing with the medium. Preparation of autoclaved bean segment medium, extraction of the enzymes, and determination of dry weight of mycelium were carried out as previously reported (16).

Assay of Enzymatic Activity

PG and Cx activity was determined viscometrically as described (16) and expressed in relative activity units (RA) (4). PL activity was determined viscometrically by mixing 2.5 ml of enzyme with 6.0 ml of 0.9% citrus pectin grade I in 0.1 M Tris-HCl buffer (pH 8.0) containing 0.002 M $CaCl_2$. In some experiments PL was also determined spectrophotometrically by measuring the increase in absorbance at 230 nm (1) in a substrate solution containing 0.3% pectin (17). This enzyme was defined as endo-PL, as the viscosity decreased rapidly (50% after 16 min.), while simultaneously the increase in absorbance was slow and linear for 12 h (17).

Results

Effect of Substrate Source and Concentration on the Induction of PG, Cx, and PL

The production of PG by *R. solani* was detected earlier than Cx when induced by a similar concentration of its respective inducers (w/v) (Fig. 1A–B). Cx activity was detected soonest in the CMC-containing medium, and latest in the fibrous cellulose-supplemented medium. Increasing CMC concentration up to 0.5% resulted in an earlier appearance of Cx. Other Cx inducers, however, only caused an increase of Cx activity. PL activity was induced to a greater extent by citrus-pectin-supplemented medium than by NaPP-supplemented medium. PG appearance on the NaPP medium preceded

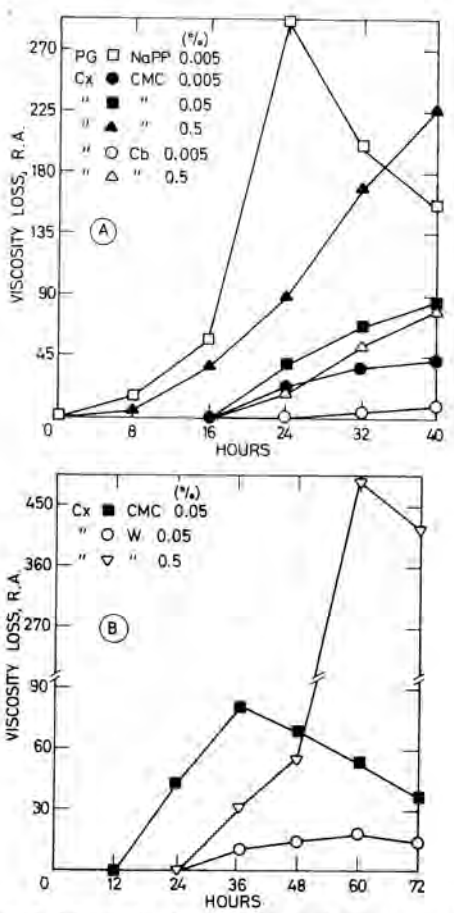

FIG. 1. Effect of substrate type and concentration on production of polygalacturonase (PG) and cellulase (Cx) by *R. solani* in Czapek medium. Inducers: sodium polypectate (NaPP), carboxymethyl cellulose (CMC), cellobiose (Cb), and Whatman fibrous cellulose powder (W).

TABLE 1

Effect of pH on the growth, time of initial appearance, and maximum production of polygalacturonase (PG), cellulase (Cx), and pectin lyase (PL) of *R. solani*[a]

Enzyme	pH[c]	Initial production		Maximum production		Growth[b]
		Hours	RA[d]	Hours	RA[d]	
PG	4.0	8	15	20	210	5.5
	6.0	12	11	32	35	5.5
	8.0	20	4.5	32	12	5.3
Cx	4.0	12	30	48	730	13.5
	6.0	12	25	72	410	10.7
	8.0	24	25	72	245	7.5
PL	4.0	—	tr[e]	—	tr[e]	23.6
	6.0	24	10	60	90	20.8
	8.0	24	25	60	160	14.5

[a]PG produced in Czapek medium supplemented with 0.005% sodium polypectate; Cx produced in Czapek medium supplemented with 0.25% carboxymethyl cellulose; and PL produced in Czapek medium supplemented with 0.5% citrus pectin.
[b]Dry weight (mg)/flask at peak time. Flasks were inoculated with 5 mg of inoculum each.
[c]Readjusted to the initial value, after each sampling, with 0.1 M HCl or NaOH.
[d]Relative activity units.
[e]Only traces of enzyme were detected.

that of PL on citrus-pectin-supplemented medium even when the concentration of the latter was increased 100-fold as compared with that of NaPP.

Effect of pH on Enzyme Production and Activity

Earliest initial production of PG was observed at pH 4.0 but lagged by 4 h at pH 6.0 as compared with pH 4.0. Cx appeared after 12 h at both pH 4.0 and 6.0. PL appeared after 24 h at both pH 6.0 and 8.0. Optimum pH values for growth, and for the activity of PG, Cx, and PL were 4.0, 5.0, 5.5, and 7.5 respectively (Table 1). This is in accordance with other reports (2, 3, 5, 6, 21).

Effect of Simultaneous Addition of Inducers or Plant Material on Sequential Enzyme Production

When enzyme production was followed in media containing pairs of inducers of PG and Cx, PG appearance always preceded that of Cx (Fig. 2). In the presence of constant concentrations of fibrous cellulose, increasing NaPP concentration resulted in a decrease in Cx activity. At a constant NaPP concentration, increasing the concentrations of fibrous cellulose had no effect on PG production. Similar results regarding the order of appearance were obtained when fibrous cellulose was replaced by either CMC or cellobiose except that those inducers repressed PG production. For example, after 30 h of incubation, the production of PG in a medium containing 0.05% NaPP plus 0.05% CMC was

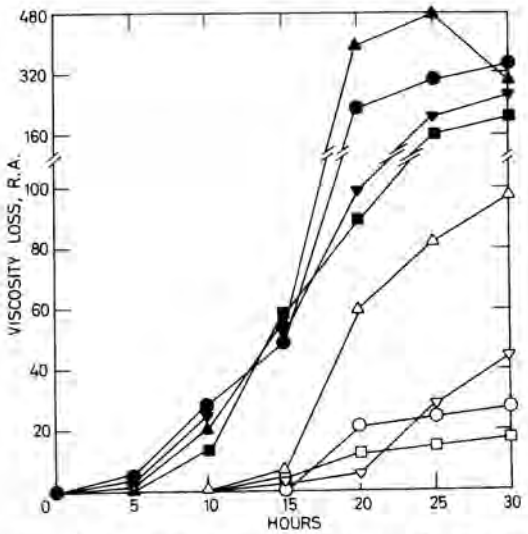

FIG. 2. Effect of sodium polypectate (NaPP) and Whatman fibrous cellulose powder (W) on sequential production of polygalacturonase (PG) and cellulase (Cx) by *R. solani* growing in Czapek medium at initial pH 4.0 in the presence of both inducers. PG is represented by full symbols and Cx by empty symbols. Inducer concentrations (%, w/v): NaPP, 0.05 + W, 0.05 (○, ●); NaPP, 0.05 + W, 0.5 (△, ▲); NaPP, 0.5 + W, 0.05 (□, ■); NaPP, 0.5 + W, 0.5 (▽, ▼).

3.4-fold higher as compared with 0.05% NaPP plus 0.5% CMC, and Cx was higher by 5.7-fold in a medium supplemented with 0.05% NaPP plus 0.05% CMC than with 0.5% NaPP plus 0.05% CMC.

When *R. solani* was grown in a modified

Czapek medium adjusted to pH 6.0 and supplemented with 0.2% NaPP plus 0.2% citrus pectin (NP medium), PG was detected 4 h earlier than PL and the appearance of its peak also preceded that of PL (Fig. 3A). Similar results were also obtained in medium containing 0.02% NaPP plus 0.02% pectin. No Cx was detected in either medium. When PL was examined spectrophotometrically the same trend was apparent. When 0.2% CMC was added to NP medium (Fig. 3B), Cx and PG were produced simultaneously, in contrast with the results previously obtained at pH 4.0 (Figs. 1, 2); PL again appeared later.

When *R. solani* was grown on autoclaved bean segments (pH 6.0) the appearance of PG was greatly retarded as compared with the other media and the pattern of its appearance was similar to that of PL and Cx, the latter being produced in very large amounts (Fig. 3C).

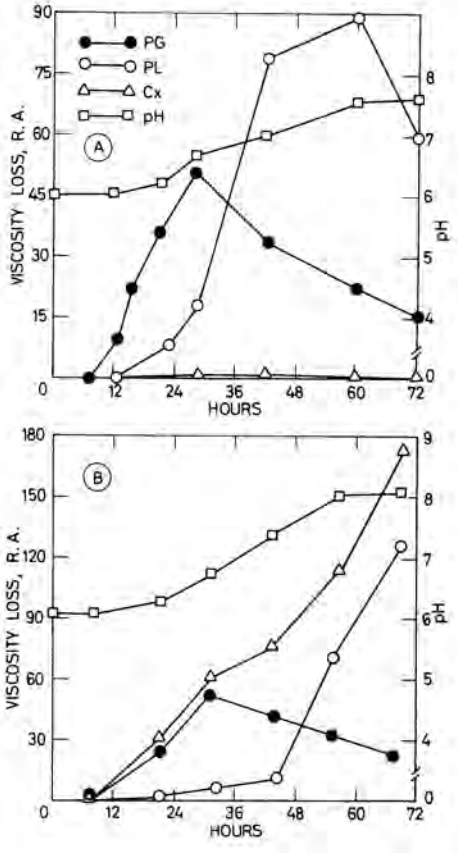

FIG. 3. Sequential production of polygalacturonase (PG), cellulase (Cx), and pectin lyase (PL) by *R. solani* at initial pH of 6.0 in Czapek medium containing (%, w/v) A, sodium polypectate (NaPP) 0.02 + pectin (P) 0.2; B, NaPP 0.2 + P 0.2 + carboxymethyl cellulose 0.2.

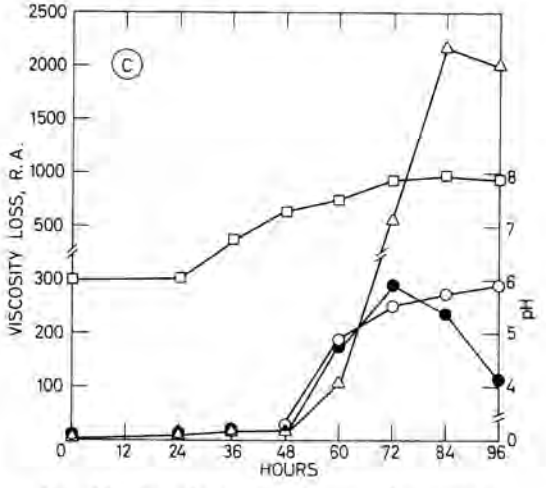

FIG. 3C. Autoclaved bean hypocotyl segments.

During growth of *R. solani* on synthetic media (Fig. 3A, B), the pH rise to 6.7–7.4 was accompanied by an increase in PL activity and a decrease in PG activity. With the autoclaved bean segment medium, however, both PG and PL appeared at high pH values (Fig. 3C).

Effect of Glucose, Cellobiose, and MGA on the Production of PG, Cx, and PL

Glucose, cellobiose, and MGA are products of Cx and PG, and may appear in the growth media as a result of their activity. They were therefore examined for their effect on the production of PG, Cx, and PL Fig. 4A–C). Production of Cx at the initial stages was markedly repressed and the appearance of Cx delayed by each of these three products. PL was not affected by any of these compounds whereas PG production was partially affected. In addition, MGA increased the production of all the enzymes in the later stages of growth.

In another experiment, the combined effect of NaPP and glucose on Cx production in the presence of CMC as an inducer was examined. Results (Fig. 5A) again showed that PG appearance precedes that of Cx, and that glucose combined with inducers has a much more pronounced repressive effect on Cx than on PG production (Fig. 5B, C), as has been shown in other experiments (Fig. 4A, B).

Discussion

The present study examines the order of appearance of PG, Cx, and PL of *R. solani* as affected by pH, type and concentration of

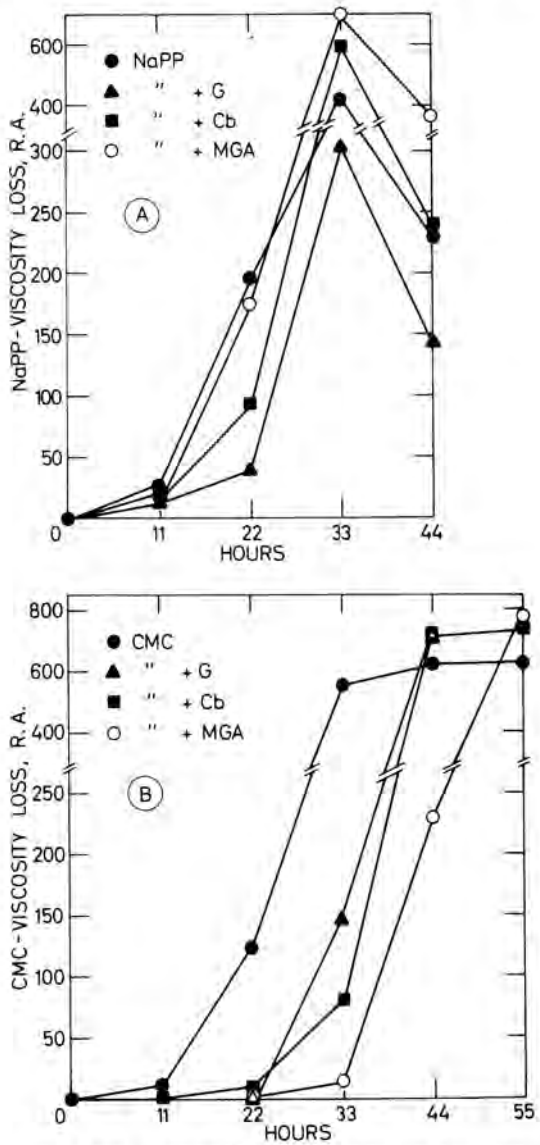

FIG. 4. Effect of 0.07 M of glucose (G), cellobiose (Cb), and monogalacturonic acid (MGA) on the production of: A, polygalacturonase; B, cellulase; and C, pectin lyase produced by *R. solani*. Erlenmeyer flasks with Czapek medium containing 0.5% of either sodium polypectate (NaPP), carboxymethyl cellulose (CMC), or pectin (P) as a sole carbon source, was inoculated with the equivalent of 7 mg (dry weight) of *R. solani*.

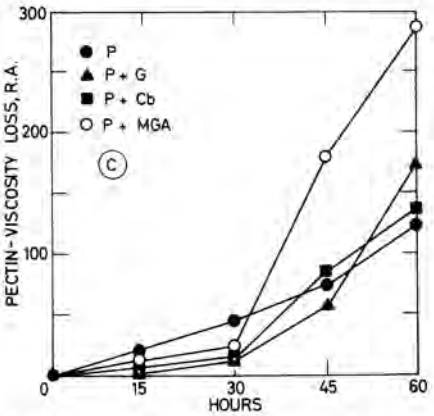

FIG. 4C. Pectin lyase produced by *R. solani*.

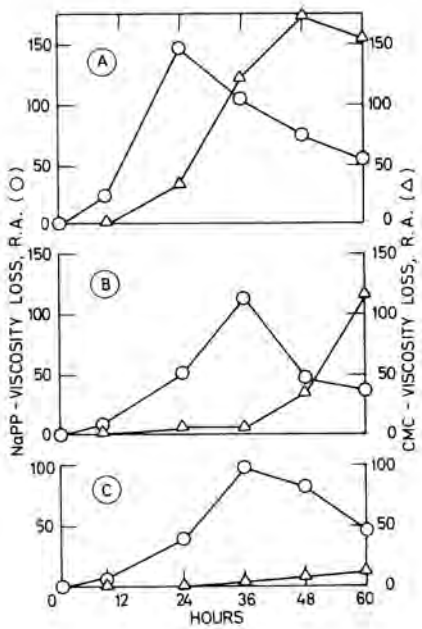

FIG. 5. Effect of glucose on the production of polygalacturonase (○) and cellulase (△) by *R. solani*. A, 0.5% sodium polypectate (NaPP) + 0.5% carboxymethyl cellulose (CMC). B, 0.5% NaPP + 0.5% CMC + 5.5×10^{-3} M glucose. C, 0.5% NaPP + 0.5% CMC + 5.5×10^{-2} M glucose.

inducer, and enzyme-degradation products. PG appeared before Cx in all substrate combinations with the exception of synthetic and plant material media at pH 6.0 when both enzymes appeared at the same time. The delayed production of Cx as compared with PG may be due to one or more of the following: (i) in the presence of both PG and Cx inducers, Cx is repressed more by NaPP than PG is by Cx inducers. (ii) Cx production is repressed more than PG by the hydrolysis products of both PG and Cx (Fig. 4). Horton and Keen (10), who showed that Cx is repressed more than PG by glucose, suggested that, in infected roots, Cx synthesis could not be expected until sugars are nearly

exhausted. This may also explain the late appearance of Cx. The pronounced increase in enzyme production at a later stage in media containing both inducers and supplemented sugars (Fig. 4) might be attributed to the exhaustion of sugars at the later stages. (iii) Even though both PG and Cx of *R. solani* are cell-bound (15), it was shown that PG is released more readily from the pathogen propagules. (iv) Because Cx appearance in *R. solani* is related to the older hyphal branching of the mycelium (11), it was detected at the later stages of growth whereas PG appeared simultaneously with propagule germination (16). (v) Cell walls of sycamore and bean hypocotyl tissues were degraded by cellulase only after pretreatment by endo-PG (B. Jurale, K. Keegstra, and P. Albersheim, cited in ref. 13).

PL production was favored at a high pH and its appearance was delayed to a later stage when a rise in the pH of the medium occurred (Fig. 3A–C). PL production was less repressed by glucose, cellobiose, and MGA than PG. These results differ from those obtained by Spalding *et al.* (22) for *Penicillium expansum* where PL production was more repressed than PG production.

When examining the sequence of enzyme production, both initial enzyme appearance and time of peak activity should be considered. The early production of a degrading enzyme at the infection site, even in small quantities, may be of importance in the early stages of infection. Thus, the early appearance of PG emphasizes its importance in the initial intercellular invasion and establishment of the parasite (10).

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1. Albersheim, P., H. Neukom, and H. Deuel. 1960. Über die Bildung von ungesättigten Abbauprodukten durch ein pektinabbauendes Enzym. Helv. Chim. Acta. **43**: 1422–1426.
2. Ayers, W. A., G. C. Papavizas, and A. F. Diem. 1966. Polygalacturonate trans-eliminase and polygalacturonase production by *Rhizoctonia solani*. Phytopathology, **56**: 1006–1011.
3. Barker, K. R., and J. C. Walker. 1962. Relationship of pectolytic and cellulytic enzyme production by strains of *Pellicularia filamentosa* to their pathogenicity. Phytopathology, **52**: 1119–1125.
4. Bateman, D. F. 1963. The "macerating enzyme" of *Rhizoctonia solani*. Phytopathology, **53**: 1178–1186.
5. Bateman, D. F. 1964. Cellulase and the *Rhizoctonia* disease of bean. Phytopathology, **54**: 1372–1377.
6. Bateman, D. F. 1967. Alteration of cell wall components during pathogenesis by *Rhizoctonia solani*. *In* The dynamic role of molecular constituents in plant-parasitic interaction. *Edited by* C. J. Mirocha and I. Uritani. The American Phytopathological Society, St. Paul, Minn. pp. 58–79.
7. Biehn, W. L., and A. E. Dimond. 1971. Effect of pectin source and sugars on polygalacturonase production by *Ceratocystis ulmi*. Phytopathology, **61**: 745–746.
8. English, P. D., J. B. Jurale, and P. Albersheim. 1971. Host–pathogen interactions. II. Parameters affecting polysaccharide-degrading enzyme secretion by *Colletotrichum lindemuthianum* grown in culture. Plant Physiol. **47**: 1–6.
9. Goodenough, P. W., and G. A. Maw. 1974. Studies on root rotting fungus *Pyrenochaeta lycopersici*: characterization of extracellular enzymes which degrade pectic compounds and factors affecting synthesis of these enzymes. Physiol. Plant Pathol. **4**: 51–62.
10. Horton, J. C., and N. T. Keen. 1966. Sugar repression of endopolygalacturonase and cellulase synthesis during pathogenesis by *Pyrenochaeta terrestris* as a resistance mechanism in onion pink root. Phytopathology, **56**: 908–916.
11. Isaac, P. K. 1964. Metabolic specialization in the mycelium of *Rhizoctonia solani* Kühn. Can. J. Microbiol. **10**: 621–622.
12. Jones, T. M., A. J. Anderson, and P. Albersheim. 1972. Host–parasite interactions: IV. Studies on the polysaccharide degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. **2**: 153–166.
13. English, P. D., A. Maglothin, K. Keegstra, and P. Albersheim. 1972. A cell wall-degrading endopolygalacturonase secreted by *Colletotrichum lindemuthianum*. Plant Physiol. **49**: 293–297.
14. Lai, M. T., A. R. Weinhold, and J. G. Hancock. 1968. Permeability changes in *Phaseolus aureus* associated with infection by *Rhizoctonia solani*. Phytopathology, **58**: 240–245.
15. Lisker, N., J. Katan, I. Chet, and Y. Henis. 1975. Release of cell-bound polygalacturonase and cellulase from mycelium of *Rhizoctonia solani*. Can. J. Microbiol. **21**: 521–526.
16. Lisker, N., J. Katan, and Y. Henis. 1974. Effect of propagule size on the in vitro production of polygalacturonase and cellulase by *Rhizoctonia solani*. Can. J. Microbiol. **20**: 1713–1721.
17. Lisker, N., and N. Retig. 1974. Detection of polygalacturonase and pectin lyase isoenzymes in polyacrylamide gels. J. Chromatogr. **96**: 245–249.
18. Mount, M. S., D. F. Bateman, and H. G. Basham. 1970. Induction of electrolyte loss, tissue maceration and cellular death of potato tissue by an endopolygalacturonate transeliminase. Phytopathology, **60**: 925–931.
19. Mullen, J. M., and D. F. Bateman. 1971. Production of an endopolygalacturonate trans-eliminase

by a potato dry-rot pathogen, *Fusarium roseum* "Avenaceum" in culture and in diseased tissue. Physiol. Plant Pathol. **1**: 363–373.

20. PATIL, S. S., and A. E. DIMOND. 1968. Repression of polygalacturonase synthesis in *Fusarium oxysporum* f. sp. *lycopersici* by sugars and its effect on symptom reduction in infected tomato plants. Phytopathology, **58**: 676–682.
21. SHERWOOD, R. T. 1966. Pectin lyase and polygalacturonase production by *Rhizoctonia solani* and other fungi. Phytopathology, **56**: 279–286.
22. SPALDING, D. H., J. M. WELLS, and D. W. ALLISON. 1973. Catabolic repression of polygalacturonase, pectin lyase, and cellulase synthesis in *Penicillium expansum*. Phytopathology, **63**: 840–844.
23. VAN ETTEN, H. D., D. P. MAXWELL, and D. F. BATEMAN. 1967. Lesion maturation, fungal development and distribution of endopolygalacturonase and cellulase in *Rhizoctonia* infected bean hypocotyl tissue. Phytopathology, **57**: 121–126.
24. WEINHOLD, A. R., and T. BOWMAN. 1974. Repression of virulence in *Rhizoctonia solani* by glucose and 3-0-methyl glucose. Phytopathology, **64**: 985–990.