Reprinted from

Canadian Journal of Microbiology

Réimpression du

Journal canadien de microbiologie

Release of cell-bound polygalacturonase and cellulase from mycelium of *Rhizoctonia solani*

N. Lisker, J. Katan, I. Chet, and Y. Henis

Volume 21 • Number 4 • 1975

Pages 521–526

National Research Council Canada

Conseil national de recherches Canada

Release of cell-bound polygalacturonase and cellulase from mycelium of *Rhizoctonia solani*[1]

N. LISKER, J. KATAN, I. CHET, AND Y. HENIS

Department of Plant Pathology and Microbiology, The Hebrew University of Jerusalem, Faculty of Agriculture, Rehovot, Israel

Accepted October 31, 1974

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.

Propagules of *Rhizoctonia solani* grown in modified Czapek's medium containing sodium polypectate or carboxymethyl cellulose as a sole carbon source produced both extracellular and cell-bound polygalacturonase (PG), and cellulase (Cx), respectively. The cell-bound enzymes can be released to various extents by shaking the germinating propagules in solutions of NaCl, KCl, phosphate buffer, Na_2EDTA (ethylenediaminetetraacetate), detergents such as Triton X-100 (octyl phenoxypolyethoxyethanol), Tween 80 (polyoxyethylene sorbitan monooleate), Celmusol, and distilled water. Sodium dodecyl sulfate (SDS) inactivated both PG and Cx but did not affect Cx activity in phosphate buffer solution. PG was more easily released by salts from the mycelium of *R. solani* than Cx. The release of both enzymes was a passive process and was not due to an osmotic effect. The amount of the cell-bound fraction was correlated with the total amount of the extracellular fraction rather than with the mycelial growth. At least one-third of the cell-bound fractions of both enzymes was found to be associated with the cell wall fraction of the mycelium.

LISKER, N., J. KATAN, I. CHET et Y. HENIS. 1975. Release of cell-bound polygalacturonase and cellulase from mycelium of *Rhizoctonia solani*. Can. J. Microbiol. **21**: 521–526.

Des propagules de *Rhizoctonia solani* qui se sont développées sur un milieu Czapek modifié contenant du polypectate de sodium ou du carboxyméthyle de cellulose comme seule source de carbone, produisent une polygalacturonase (PG) extracellulaire et liée à la cellule, et une cellulase (Cx) respectivement. Les enzymes attachées à la cellule peuvent être libérées jusqu'à un certain point par agitation des propagules en germination dans des solutions de NaCl, KCl, tampon phosphate, Na_2EDTA, des détergents Triton X-100, Tween 80, Celmusol, et l'eau distillée. Le sulfate dodécyl de sodium (SDS) inactive la PG et la Cx, mais n'affecte pas l'activité de la Cx dans une solution tamponnée au phosphate. La PG est plus facilement libérée par les sels du mycélium de *R. solani* que la Cx. La libération des deux enzymes est un processus passif et n'est pas due à un effet osmotique. La quantité de la fraction retenue à la cellule est en corrélation avec la quantité totale de la fraction extracellulaire et non avec la croissance mycélienne. Au moins le tiers des fractions retenues à la cellule chez les deux enzymes est associé avec la fraction de la paroi cellulaire du mycélium.

[Traduit par le journal]

Introduction

Polygalacturonase (PG, poly-α-1,4 galacturonide glycanohydrolase, EC. 3.2.1.15) and cellulase (Cx, β-1,4 glucan glucanohydrolase, EC. (3.2.1.4) play an important role in *Rhizoctonia* damping-off diseases during the early stages of pathogenesis; whereas PG promotes the intercellular spread of the pathogen (4), Cx favors its penetration into the cell (3).

In many pathogenic fungi, such as *Colletotrichum* (8), *Pyrenochaeta* (10), *Fusarium* (12), and also *Rhizoctonia* grown in various media (unpublished data), PG production was found to precede that of Cx. Van Etten *et al.* (17) reported that at the early stages of bean infection by *Rhizoctonia*, PG was produced at higher amounts than Cx. These findings were based on measurements of the extracellular enzymes released by the fungi into their environment during growth. PG was reported to be bound by salt and disulfide linkages to the cell components outside the membrane in *Geotrichum candidum* (1), whereas Cx was reported to be partially bound to the mycelium of *Fomes annosus* (7), but also to be present inside the mycelium of *Aspergillus fumigatus* (16). BeMiller *et al.* (5) presented a model with *Diplodia zeae* in which D-glucose in the medium was responsible for the binding of Cx to fungal cells.

In the present work, the binding, release, and localization of PG and Cx of *R. solani* were

[1]Received August 28, 1974.

studied in an attempt to explain the pattern of appearance of extracellular PG and Cx activity during growth of the fungus.

Materials and Methods

Preparation of Propagules

A pathogenic strain of *Rhizoctonia solani* Kuehn (14) was grown on liquid yeast extract medium (9) in standing cultures at 27C for 7 days. The mycelial mat was removed and homogenized for 1 min with a sterile solution containing 0.1 M KCl and 0.02 M citrate buffer at pH 4.8 in a Waring blendor. The resulting homogenized fragments, referred to as propagules, were passed through plastic sieves of 250- to 500-μ size and then washed with the same buffer solution (14). The propagules on the sieves were further transferred into a solution containing the mineral salts of Czapek's medium and washed twice by decantation. The KCl–buffer solution was used for washing the propagules because in preliminary experiments it was found that if the propagules were washed with distilled water and transferred to a fresh medium, a considerable amount of PG was released upon transfer. The whole process of propagule preparation was carried out aseptically at 4C.

Production of Germinating Propagules for Enzyme-release Experiments

Propagules equivalent to 5 mg dry weight (80C) were used to inoculate 15 ml of medium in 250-ml flasks. The medium (pH 4.0) consisted of Czapek's salt solution in which the sole carbon source was either 0.5% sodium polypectate (NaPP, Sigma Chemical Co.) as PG inducer, or 0.5% carboxymethyl cellulose (CMC, BDH) as Cx inducer. To prevent bacterial contamination, 250 μg/ml of chloramphenicol (Abic, Israel) was added to the medium. A previous study (14) has shown that chloramphenicol has no significant effect on enzymatic activity in this system. The flasks were incubated at 27C in the dark in a shaker (90 strokes/min). After incubation, the germinating propagules were filtered through a 40- to 50-μ plastic sieve, and washed four times with 100 ml of cold distilled water to free the cells from any adhering enzyme. These propagules were used for the enzyme release tests.

Release of Cell-bound Enzymes

To release PG or Cx enzymes, the following chemical reagents were used: disodium salt of EDTA (ethylenediaminetetraacetate, Sigma), 2-mercaptoethanol (Sigma), SDS (sodium dodecyl sulfate, Sigma), Triton X-100 (octyl phenoxy polyethoxyethanol, Sigma), Tween 80 (polyoxyethylene sorbitan monooleate, Sigma), Celmusol (Melle Bezons, France), NaCl, KCl, and K-phosphate buffer solution. The washed germinating propagules were vigorously shaken for 1 h at 4C in 100-ml flasks containing 10 ml of the enzyme-releasing solution (pH 5.2) and filtered through a 40- to 50-μ plastic sieve. After assaying the filtrate for PG and Cx activity, the dry weight of the mycellium from which the enzymes were released was determined by drying the mycelium in an oven at 80C to a constant weight.

Enzymatic Activity

Enzymatic activities were tested by following the decrease in viscosity of the reaction mixture with an Ostwald viscometer at 30C (14). The reaction mixture contained 2.5 ml of filtrate and 6 ml of substrate (1.2% NaPP for PG activity or 0.9% CMC for Cx activity in 0.05 M citrate buffer at pH 4.8). Results were expressed either as percentage decrease of viscosity per time unit or as relative enzymatic activity (RA), which was defined as 1000 × the reciprocal of time (in minutes) required for 50% reduction in the viscosity of the substrate (2).

Preparation of Cell Wall (6)

Germinating propagules were washed thoroughly with distilled water, homogenized in an Omnimixer (Sorvall), and treated in a MSE 500W ultrasonic disintegrator at an operation frequency of 20 kilocycles/s, for 10 min. During treatment the vessel containing the sample was immersed in crushed ice. Coarse particles were centifuged off at 800 *g* for 10 min and the remaining fine particles were further precipitated at 12 000 *g* for 15 min. The cell wall precipitate was then washed with distilled water until no residual glucose or nucleic acids could be detected in the supernatant. Glucose was determined with a glucose oxidase reagent (Glucostat, Worthington Biochemical Corp., Freehold, New Jersey). Nucleic acids were assayed with a Techtron UV spectrophotometer (Varian) at 260 nm. Examination of this fraction under a Zeiss phase-contrast microscope revealed that it was composed entirely of hyphal cell walls and did not contain any unbroken hyphae.

Results

Effect of Buffer and Salt Solutions on the Release of PG and Cx from R. solani Mycelium

Germinated propagules of *R. solani* were washed with distilled water, shaken in phosphate buffer solutions, and the filtered supernatant was assayed for the activity of the released PG and Cx. Enzymatic activity in the supernatant was found to increase with increasing phosphate buffer concentration up to 0.3 M. This was found with both enzymes; however, PG release was higher than that of Cx at all buffer concentrations tested (Fig. 1). In other experiments, release of cell-bound enzymes by various salt concentrations, as compared with distilled water, was studied. Whereas the reduction in viscosity by PG and Cx released upon shaking in distilled water was 9.5% and 5.4%, respectively, after 20 min of incubation, the amounts of PG and Cx released upon shaking in 0.3 M KCl solution were 440% and 174%, respectively (when activity released by distilled water = 100%). Addition of 0.3 M KCl to 0.1 M phosphate buffer solution increased the released enzymatic activity to 707% and 228% of PG and Cx, respectively. This release could not be attributed to osmotic shock, since when glucose (0.1 M) was used instead of 0.05 M KCl, PG and Cx activities, as compared with

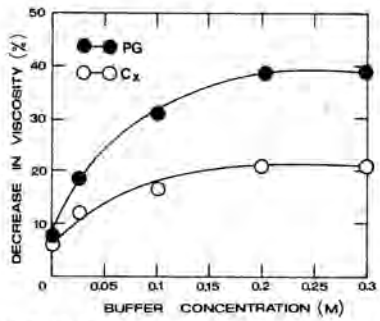

FIG. 1. Effect of phosphate buffer concentration (pH 5.2) on release of polygalacturonase (PG) and cellulase (Cx) from germinating propagules of *Rhizoctonia solani*. Enzyme activity is expressed as percentage decrease in viscosity after 20 min of incubation.

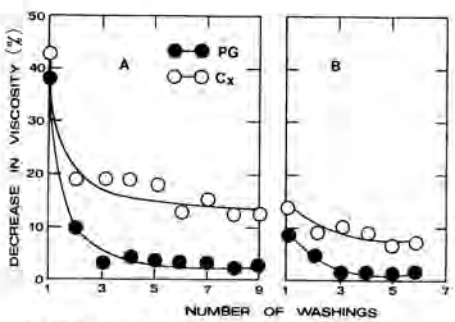

FIG. 2. Effect of number of washings on polygalacturonase (PG) and cellulase (Cx) released from germinating propagules of *Rhizoctonia solani* (A) by buffer phosphate 0.1 M pH 5.2 + KCl 0.3 M, and (B) by distilled water. Enzyme activity is expressed as percentage decrease in viscosity after 75 min of incubation.

water, were 122% and 116%, respectively. Sodium chloride (0.1 M) released 30% more PG as compared with 0.1 M KCl, whereas similar amounts of Cx were released with both salts.

The effect of washing with buffer–KCl solutions or distilled water on the release of PG and Cx from the propagules was examined in relation to number and duration of treatments. Again, the results showed that PG was more easily released than Cx, as three washings were sufficient for the release of most of the bound PG (Fig. 2A, B). Both enzymes were less easily removed by successive washings with distilled water as compared with buffer–KCl. Transfer of the mycelium to a buffer–KCl solution after the seventh washing with distilled water resulted in an increased release of the remaining cell-bound PG and Cx into the solution, with a

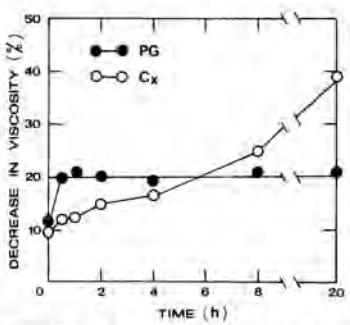

FIG. 3. The effect of shaking duration of *Rhizoctonia solani* germinating propagules on the release of polygalacturonase (PG) and cellulase (Cx). Shaking was done in a phosphate buffer, 0.1 M pH 5.2 + KCl 0.3 M solution. Propagules grown on sodium polypectate – Czapek (pH 4.0) and on carboxymethyl cellulose – Czapek (pH 4.0) were used to follow the release of PG and Cx, respectively. Enzyme activity is expressed as percentage decrease in viscosity after 65 min of incubation.

resulting decrease in viscosity of 28% and 22%, respectively.

The effect of the duration of the washing treatment on the release of PG and Cx into buffer–KCl solution and water is shown in Fig. 3. Whereas shaking the mycelium for 30 min in buffer–KCl solution released most of its cell-bound PG, release of Cx continued throughout the 20-h period of the experiment. Similar results were obtained when the fungus was grown on Whatman fibrous cellulose powder (CF1) instead of CMC for Cx production. The release of Cx by distilled water followed a trend similar to that of the buffer–KCl solution, but at a lower rate. The pattern of continuous release of Cx could not be attributed to enzyme activation, since the incubation of an extracellular enzyme solution with the buffer–KCl solution for 20 h at 4C resulted in 13% decrease in its activity. Neither could this pattern be related to possible *de novo* enzyme synthesis during the incubation at 4C, since the addition of 50 µg/ml actidione (Cycloheximide, NBC) did not alter the activity pattern throughout the incubation period.

Effect of Growth Period on the Release of Cell-bound PG and Cx

Propagules were incubated in Czapek's medium for 84 h. Samples were withdrawn at intervals and assayed for extracellular and cell-bound PG and Cx release as well as for mycelial growth. The results (Fig. 4A, B) show that the release of cell-bound PG and Cx from the grow-

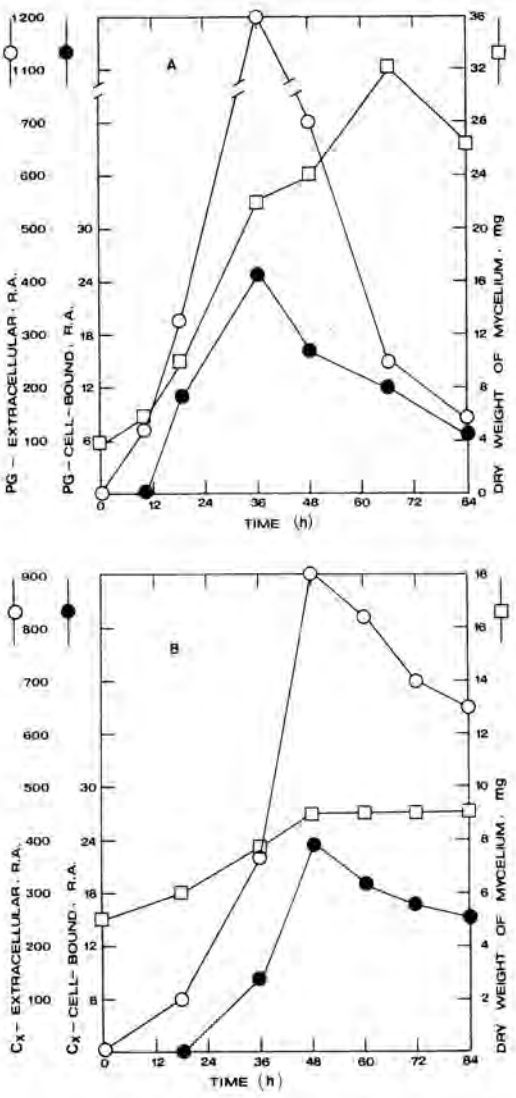

FIG. 4. Relationship between mycelial growth (□), extracellular (○) and cell-bound (●) activities of polygalacturonase (PG) and cellulase (Cx) of *Rhizoctonia solani*. Mycelial growth is expressed as dry weight. Enzymatic activity is expressed in units of relative activity (R.A.). (A) PG; (B) Cx.

ing propagules by buffer–KCl is related to the total activity of the extracellular enzymes released in the growth medium rather than to the mycelial dry weight. With both PG and Cx, peaks of activity of the cell-bound and of the extracellular enzymes appeared simultaneously. Addition of 50 μg/ml actidione to the growth medium prevented the appearance of both cell-bound and extracellular PG and Cx.

Release of PG and Cx by Various Chemical Agents

The effects of various chemical agents on the release of PG and Cx are summarized in Table 1. Sodium EDTA was found to increase the activity of PG and Cx in the filtrate by 457% and 166%, respectively. Combinations of EDTA, phosphate buffer, and KCl were found to release the enzymes at rates similar to those obtained with phosphate buffer and (or) KCl alone. In another experiment, 0.1 M Na_2EDTA was compared with 0.1 M NaCl for the capacity to release PG. Both solutions had the same conductivity; thus they contained the same Na^+ concentration. Na_2EDTA was found to release 24% more PG than did NaCl.

The detergents Tween 80, Triton X-100, and Celmusol caused only a slight release of PG but had a more pronounced effect on Cx. SDS, which inactivates certain pectin esterases (11), also inactivates PG and Cx. However, the inhibiting effect of SDS on Cx was not observed in the buffer solution. Neither PG nor Cx was found to be released by mercaptoethanol.

Localization of Cell-bound PG and Cx in R. solani Mycelia

Germinating propagules and their corresponding purified cell wall preparations were examined for their ability to release PG and Cx when incubated in a 0.3 M KCl–phosphate buffer solution (pH 5.2). Of the total amounts of 325 units and 153 units, 36% of the PG activities and 30% of the Cx activities were found to be bound to the cell wall fraction.

Discussion

In the present work it was demonstrated that PG and Cx produced by *R. solani* appear in both extracellular and cell-bound forms. This is in agreement with observations made using other microorganisms (1, 5, 7, 13, 15). The cell-bound enzymes were released from *R. solani* mycelia in varying degrees by distilled water, salt solutions, Na_2EDTA, and detergents. Cx seemed to be bound to cells stronger than PG since its release required higher concentrations of buffers and more intensive washings for a much longer time, and was not influenced by the type of inducer in the growth medium. The apparent delay in appearance of Cx in the media, as compared with PG in various fungi (8, 10, 12) and in *R. solani* (unpublished data), may also be attributed to a slower release of this enzyme, already formed and attached to the cells.

TABLE 1

The effect of chemical agents on the activity of polygalacturonase (PG) and cellulase (Cx) of *Rhizoctonia solani* and on the release of the enzymes from the mycelium

Chemical agent[a]	PG		Cx	
	Released enzyme[b]	Effect on enzymatic activity[c]	Released enzyme[b]	Effect on enzymatic activity[c]
B, pH 5.2, 0.1 M	505	126	161	116
B + 0.3 M KCl	707	122	228	118
Na_2EDTA 0.1 M	457	122	166	110
Na_2EDTA + B	666	122	153	118
Na_2EDTA + B + 0.3 M KCl	—[d]	—[d]	189	123
T_{80} 1%	135	112	151	110
T_{80} + B	554	120	200	118
Tx 1%	115	110	148	112
Tx + B	509	122	208	120
C 1%	132	110	161	110
C + B	500	123	210	116
SDS 1%	0	0	18	8
SDS 0.1% + B[e]	0	15	155	103
M, 0.2 M	100	98	112	104
M + B	425	128	188	120
M + B + 0.3 M KCl	701	130	182	124

[a] B = phosphate buffer, T_{80} = Tween 80, Tx = Triton X-100, C = celmusol, M = mercaptoethanol.
[b] Percentage enzymatic activity as compared with that observed in mycelium shaken in distilled water (100%), which showed a reduction in viscosity for PG and Cx of 9.5% and 5.4% respectively. Incubation time of supernatant with substrate was 20 min.
[c] Measured by comparing the activity of an enzyme solution in the presence and absence of the tested chemical agent. Decrease in viscosity of 25.6% for PG and 31.0% for Cx after 20 min of incubation with substrate was taken as 100%.
[d] A great nonenzymatic increase in viscosity did not allow viscometric measurements to be carried out.
[e] This concentration was used because when 1% SDS was mixed with phosphate buffer a heavy precipitate occurred.

At least one-third of the cell-bound PG and Cx that could be released from intact cells is located in the purified cell walls. In spite of their effect on the cell membrane, the detergents caused a relatively small release of these enzymes. Since cell-bound PG and Cx were not released by the sulfhydryl reagent 2-mercaptoethanol, it appears that these enzymes are not bound to cells by S—S bonds.

Conditions required for the release of cell-bound PG of *R. solani* were not the same as those found by Barash and Klein (1) for the release of PG of *G. candidum*. However, different binding properties of similar enzymes to cells of different fungi have also been demonstrated for invertase and acid phosphatase in *Saccaromyces fragilis* and *S. cerevisiae* (18). The release of Cx in other organisms was reported to be achieved by polygalacturonic acid or lignosulfonic acid (5), by EDTA–lysozyme in isotonic solution of sucrose (15), and by lysis with desoxycholate (13).

The relative activities of the cell-bound fractions of both enzymes could be correlated with the total amounts of the enzymes released into the medium during growth rather than with the age of the mycelia. Similarly, Cowling and Kelman (7) found that the amount of Cx bound to the fungus *F. annosus* was related directly to the total Cx activity in the cultures.

The ratio between the amounts of the cell-bound and extracellular fraction of PG and Cx varied during the growth period of *R. solani*. This ratio was shown by Suzuki *et al.* (15) to be affected in *Pseudomonas fluorescens* by the type of the carbon source used in the growth medium.

The pattern of appearance of extracellular PG and Cx produced by *R. solani* in growth media may depend on the rates of their production and release from the cell walls. Release of cell-bound PG and Cx of *R. solani* is affected by the ionic composition of the medium. As these enzymes did not appear in the growth medium supplemented with actidione, it may be concluded that both the cell-bound and the extracellular fractions were newly synthesized, rather than formerly present, enzymes.

The release of cell-bound enzymes is a passive

process since it occurs at low temperature or in the presence of actidione. This release is probably enhanced by plant exudates and could be of importance for the pathogen at the initial infection stages.

1. BARASH, I., and L. KLEIN. 1969. The surface localization of polygalacturonase in spores of *Geotrichum candidum*. Phytopathology, **59**: 319–324.
2. BATEMAN, D. F. 1963. The "macerating enzyme" of *Rhizoctonia solani*. Phytopathology, **53**: 1178–1186.
3. BATEMAN, D. F. 1964. Cellulase and the *Rhizoctonia* disease of bean. Phytopathology, **54**: 1372–1377.
4. BATEMAN, D. F., and R. L. MILLAR. 1966. Pectic enzymes in tissue degradation. Annu. Rev. Phytopathol. **4**: 119–146.
5. BEMILLER, J. N., D. O. TEGTMEIER, and A. J. PAPPELIS. 1969. Constitutive cellulolytic enzymes of *Diplodia zeae*. *In* Cellulases and their applications. Adv. Chem. Ser. **95**: 188–196.
6. CHET, I., Y. HENIS, and R. MITCHELL. 1967. Chemical composition of hyphal and sclerotial walls of *Sclerotium rolfsii* Sacc. Can. J. Microbiol. **13**: 137–141.
7. COWLING, E. G., and A. KELMAN. 1963. Cellulolytic activity of monobasidiospore and tissue isolates of *Fomes annosus*. Phytopathology, **53**: 873.
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. HENIS, Y., and Y. BEN-YEPHET. 1970. Effect of propagule size of *Rhizoctonia solani* on saprophytic growth, infectivity, and virulence on bean seedlings. Phytopathology, **60**: 1351–1356.
10. HORTON, J. C., and N. T. KEEN. 1966. Sugar repression of endopolygalacturonase and cellulase synthesis during pathogenesis by *Pyrenochaeta terrestris* as a resistence mechanism in onion pink root. Phytopathology, **56**: 908–916.
11. HULTIN, H. O., and A. S. LEVINE. 1963. On the occurrence of multiple molecular forms of pectinesterase. Arch. Biochem. Biophys. **101**: 396–402.
12. JONES, T. M., A. J. ANDERSON, and P. ALBERSHEIM. 1972. Host–pathogen interactions. IV. Studies on the polysaccharide-degrading enzymes secreted by *Fusarium oxysporum* f. sp. *lycopersici*. Physiol. Plant Pathol. **2**: 153–166.
13. KING, K. W. 1959. Activation and cell-surface localization of certain β-glucosidases of the ruminal flora. J. Dairy Sci. **42**: 1848–1856.
14. 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.
15. SUZUKI, H., K. YAMANE, and K. NISIZAWA. 1969. Extracellular and cell-bound cellulase components of bacteria. *In* Cellulases and their applications. Adv. Chem. Ser. **95**: 60–82.
16. TASHPULATOV, ZH. 1966. Cellulolytic enzymes of thermophilic and mesophilic strains of *Aspergillus fumigatus*. Mikrobiologiya, **35**: 27–34; Chem. Abstr. 1966, **64**: 16322.
17. 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. Phytopathology, **57**: 121–126.
18. WEIMBERG, R., and W. L. ORTON. 1966. Elution of exocellular enzymes from *Saccharomyces fragilis* and *Saccharomyces cerevisiae*. J. Bacteriol. **91**: 1–13.