

The Role of Chitinase of *Serratia marcescens* in Biocontrol of *Sclerotium rolfsii*

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ABSTRACT

Ordentlich, A., Elad, Y., and Chet, I. 1988. The role of chitinase of *Serratia marcescens* in biocontrol of *Sclerotium rolfsii*. *Phytopathology* 78:84-88.

Serratia marcescens was grown on cell wall of *Sclerotium rolfsii* and its components, chitin and laminarin, as a sole carbon source. A culture filtrate, possessing chitinolytic activity, was obtained and incubated with different substrates, including *S. rolfsii* cell wall, dry mycelium, and washed mycelium. A release of *N*-acetyl-D-glucosamine at rates of 1-5.2 chitinase

units indicated substrate degradation. Degradation of hyphae of the plant pathogen was observed by light and scanning electron microscopy. Crude chitinase caused very swift swelling at the hyphal tips of the fungus. Sixty-three percent of the cells of the hyphal tips lysed.

Antagonism, responsible for some types of biological control, may operate by antibiosis, competition, or exploitation (20). Exploitation is subdivided into predation and direct parasitism. Parasitism relies on lytic enzymes for the degradation of cell walls of pathogenic fungi. Cell walls of Basidiomycetes and Ascomycetes contain chitin (2,7). Several bacteria and fungi have been shown to

produce hydrolytic enzymes after induction by the appropriate substrates (3,4,21). Mitchell and Alexander (14) have demonstrated biological control of *Fusarium* spp. and *Pythium* sp. by bacteria that degrade the cell wall of these plant pathogens. Mitchell and Hurwitz (16) protected tomato plants against damping off, caused by *Pythium aphanidermatum* (Edson) Fitzpatrick, by lytic *Arthrobacter*. Koths and Gunner (12) and Sneh (23) also demonstrated biological control of disease caused by *Fusarium* on carnation by using a chitinolytic *Arthrobacter* sp.

Enterobacter cloacae (Jordan) Hormaeche & Edwards controlled *Pythium* in peas, sugar beet, and cucumber, probably by degrading the mycelium (10). Campbell (5) and Campbell and Ephgrave (6) showed that hyphal lysis, induced by *Bacillus* sp. in soil was involved in a biological control of *Gaeumannomyces graminis* (Sacc.) v. Arx & Olivier.

Serratia marcescens Bizio, isolated from the rhizosphere of plants grown in soil infested with *Sclerotium rolfii* Sacc., was found to be an effective biocontrol agent against this pathogen and *Rhizoctonia solani* Kühn, under greenhouse conditions (19). The objective of this study was to determine whether the chitinolytic enzymes of *S. marcescens* are involved in the biological control of *S. rolfii*.

MATERIALS AND METHODS

S. marcescens was isolated from rhizosphere of plants and identified by the Analytical Profile Index (API) system (La Balme les Grottes 38390, Montalieu, Vercieu, France). Identification was verified by Bergey's Manual of Determinative Bacteriology. The fungus *S. rolfii*, isolated from diseased plants, was grown on a synthetic medium (SM; 18).

Infestation of greenhouse soils. *S. rolfii* was grown on SM for 3 wk. Sclerotia produced by the fungus were separated from the medium and dried in a desiccator for 48 hr. In biocontrol experiments with beans, 50 mg of sclerotia was mixed with 1 kg of soil.

Bacteria were applied into the soil as a water suspension after washing the cells by centrifugation and resuspending them in distilled water. Polypropylene boxes (7 × 19 × 14 cm) were filled with infested soil, planted with 10 bean seeds (*Phaseolus vulgaris* L. 'Brittle Wax') each, and irrigated once a day, and the number of diseased seedlings was recorded.

All experiments were conducted under greenhouse conditions, at 28–32 C, in six replicates. Sandy loam soil (pH 7.2) was used throughout this work. Soil sterilization was carried out in an autoclave, on three consecutive days, for 1 hr each time. Seeds were planted on the day of soil treatment with bacteria. Experiments were repeated three times.

Enzymatic activity tests. The cell wall degrading enzymes β -N-acetyl-D-glucosaminidase (chitinase complex) and exo β -1,3-D-glucosidase (glucanase) were assayed in *S. marcescens* filtrates. The bacterium was grown on colloidal chitin (22), *S. rolfii* mycelium or cell wall (CW), laminarin (Sigma Chemical Company, St. Louis), laminarin and chitin, or N-acetyl-D-glucosamine (GlcNac) (Sigma) as a sole carbon source. Tween 20 (0.05%) was either added to the liquid growth media before inoculation with bacteria or at the end of bacterial growth to enhance enzyme availability, or not at all (17).

S. rolfii was grown in liquid SM, at 30 C, for 72 hr before mycelium isolation. The mycelium was aseptically washed. Enzymatic tests and preparation of fungal cell walls were carried out according to previous publications (8,9). Crude enzyme solution was mixed with the following substrates: *S. rolfii* CW, fresh or dried mycelium, or colloidal chitin at a concentration of 0.02% (dry matter). Specific activity (chitinase units, CHU) was determined as micromoles of N-acetyl-D-glucosamine per milligram of protein per hour, and glucanase units (GU) as micromoles of glucose per milligram of protein per hour (9).

Lysis of hyphal tips. Agar disks, bearing *S. rolfii* mycelium (10 mm in diameter), were sprayed with extracellular filtrate from *S. marcescens* cultures containing chitinase with a specific activity of 5 CHU. Hyphal tip lysis was observed under a light microscope.

Scanning electron microscopy. Cellophane membranes bearing mycelial samples were taken from the interacting regions of *S. rolfii* and *S. marcescens*. The samples were fixed for 48 hr in a closed vessel containing two petri dishes, one with 5% OsO₄ (Sigma) in 0.1 M phosphate buffer, pH 7.4, and the other with 25% glutaraldehyde (Sigma), air dried for 24 hr, coated with gold palladium in a Polaron E5150 (Polaron Equipment Ltd., Watford, England) (1), and observed in a scanning electron microscope (JEOL JSM 35C).

RESULTS

Disease development in soil. Disease development in *S. marcescens*-treated sterilized or raw soil was slower than in the nontreated controls (Fig. 1).

Production of extracellular hydrolytic enzymes. Glucanase and chitinase produced by *S. marcescens* in the presence of different carbon sources are presented in Table 1. Chitinase was detected in media containing colloidal chitin, *S. rolfii* CW, GlcNac, or laminarin with chitin. Glucanase was tested in media containing laminarin, *S. rolfii* CW, and laminarin with chitin.

Glucanase activity was observed at a very low level (0.6–1.6 GU). Chitinase activity was observed in media containing chitin or chitin with laminarin as carbon sources. Tween 20 added to these culture media substantially increased (by 100%) chitinase production. A tenfold increase was observed by adding Tween 20 to media containing *S. rolfii* CW as a sole carbon source. The high yield of extracellular chitinase in the presence of chitin (4.9 CHU) coupled with the fact that a small yield was observed in the presence of GlcNac and other carbon sources indicates that the enzyme is inducible.

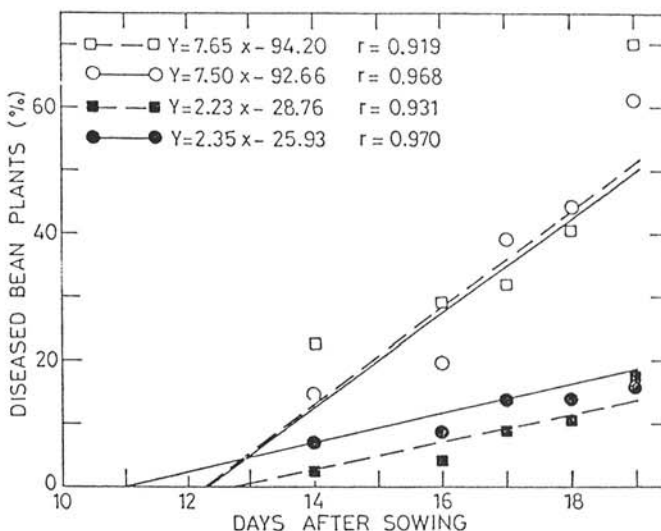


Fig. 1. Development of disease in beans caused by *Sclerotium rolfii* in sterilized soil (---) and nonsterilized soil (—). Each soil was treated with 10^6 cells per gram of *S. marcescens* (●, ■) or not treated (○, □).

TABLE 1. Effect of various carbon sources on chitinase and glucanase production by *Serratia marcescens*^a

Carbon source (0.02%)	Chitinase specific activity (CHU) ^b			Glucanase specific activity (GU) ^b		
	Without Tween 20	With ^c Tween 20	Tween 20 ^c at end	Without Tween 20	With ^c Tween 20	Tween 20 ^c at end
	Colloidal	2.16	4.94	4.15	... ^d	...
Laminarin	0	0	0
Laminarin with chitin	2.35	3.10	5.20	0.59	1.39	1.6
<i>S. rolfii</i> cell wall	0.10	1.20	1.05	0	0	0
N-acetyl-D-glucosamine	0.12	0	0	0

^a *S. marcescens* was grown in liquid synthetic medium containing the various carbon sources for 72 hr.

^b Specific activity (chitinase units, CHU) was determined as micromoles of N-acetyl-D-glucosamine per milligram of protein per hour and glucanase units (GU) as micromoles of glucose per milligram of protein per hour.

^c Tween (0.05%) was added to the medium at the time of inoculation of medium with bacteria or 2 hr before termination of incubation.

^d Not tested.

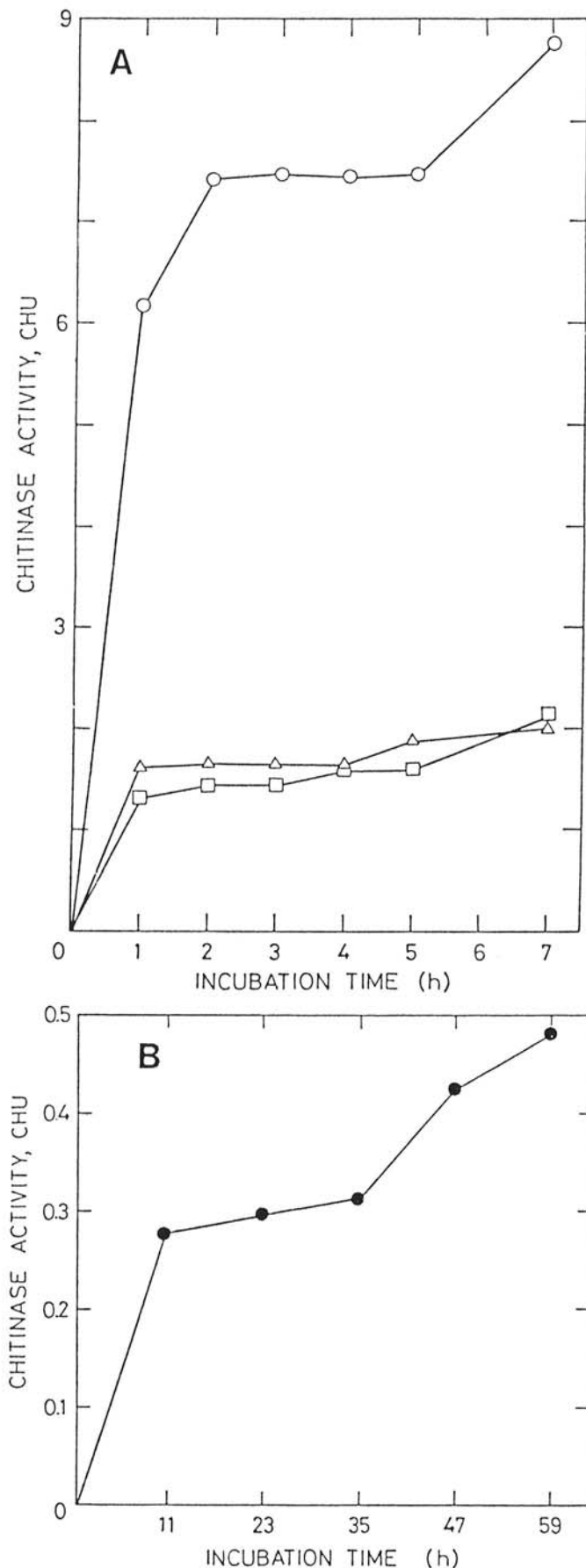


Fig. 2. Release of *N*-acetyl-D-glucosamine from: A, Chitin (o), *Sclerotium rolfsii* cell wall (Δ), dry mycelium (□) and B, fresh mycelium of *S. rolfsii* (●). Enzyme filtrate possessing activity of 7.4 chitinase units originated from 3-day-old culture of *Serratia marcescens* that was grown on 0.02% colloidal chitin.

Release of GlcNac from cell wall components. *S. marcescens* was grown in a medium containing colloidal chitin. The extracellular culture filtrate was collected and tested for its ability to release GlcNac from the following substrates: chitin, *S. rolfsii* CW, and living or dry mycelium (Fig. 2). The highest amount of released monomer was observed when the extract was incubated with colloidal chitin for 7 hr (8.7 CHU). Lower activities were observed when the extract was incubated with *S. rolfsii* CW or dry mycelium for 7 hr (2 and 2.2 CHU, respectively). With *S. rolfsii* living mycelium as the sole carbon source, only 0.5 CHU was released after 59 hr of incubation.

Lysis of *S. rolfsii* hyphal tips. Spraying solutions of extracellular culture filtrate of *S. marcescens*, grown in a medium containing chitin as a sole carbon source, on growing mycelia of *S. rolfsii* caused very swift swelling at the hyphal tips (Fig. 3). After 135 min, 63% of the hyphal tip cells were lysed. However, only 10% of the hyphal tips burst when the mycelium was treated as above with boiled extracellular extract (Fig. 4).

Microscopical observations. Scanning electron microscopical observations revealed the degradation of *S. rolfsii* mycelium. Holes were clearly observed on the hyphae in the regions of interaction with the antagonistic bacterium (Fig. 5).

Washed mycelium of *S. rolfsii* incubated with *S. marcescens* in liquid SM containing no additional carbon source was completely degraded within 7 days. Mycelium incubated without the bacterium was not degraded during the same period.

DISCUSSION

We have tested the antagonistic bacterium, *S. marcescens*, in sterilized soil to determine whether its mechanisms of biocontrol are the result of a direct or indirect attack on the pathogen, *S. rolfsii*. There was no significant difference ($p = 0.05$) between disease control in sterilized and raw soils. In both, disease levels were significantly higher than in the *Serratia* treatments. It can therefore be concluded that the interaction between *S. marcescens* and *S. rolfsii* in soil is direct and does not involve interaction with the general population of microorganisms in soil.

Microorganisms capable of lysing other organisms are widespread in natural ecosystems (11). They seem to play an important role in microbial equilibrium in soil and can serve as a powerful tool for microbiological control. Mitchell and Alexander (14,15) demonstrated how cell wall-lytic bacteria added to soil control *Fusarium* spp. (12,23) and *Pythium* (10,16) by destroying

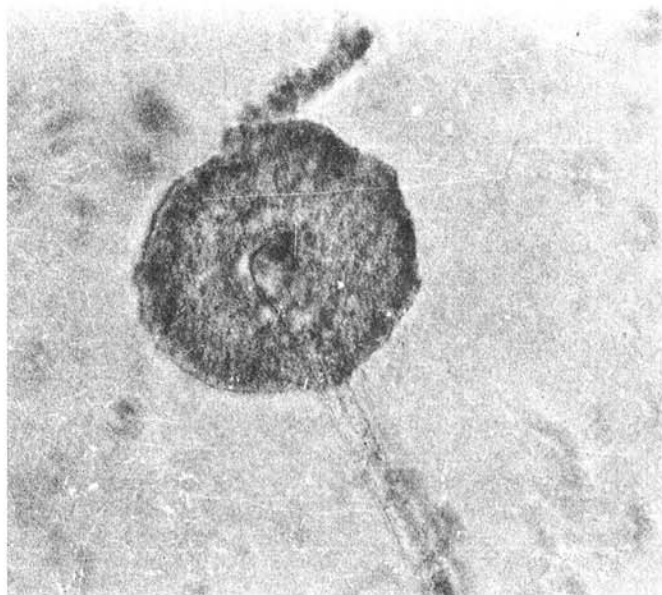


Fig. 3. The effect of *Serratia marcescens* filtrate on growing mycelium of *Sclerotium rolfsii*. Swift swelling at the hyphal tips were observed. (Light micrography $\times 400$)

fungal mycelium through their lytic activity. Campbell and Ephgrave (6) showed that hyphal lysis, induced by *Bacillus* sp. in soil, was involved in the mechanism of biological control of *G. graminis*.

We found that *S. marcescens* released a chitinolytic enzyme when grown on different polymers, such as chitin, laminarin, or *S. rolfssii* cell wall as a carbon source. This enzyme was found to be inducible. Several works show that efficient parasitic biocontrol agents secrete extracellular lytic enzymes capable of degrading chitin and laminarin (8,9,14,16,23). *S. marcescens* did, indeed, produce extracellular chitinase when grown on colloidal chitin as a sole carbon source. When this filtrate was incubated with *S. rolfssii* cell wall or mycelium, GlcNac was released, indicating the degradation of these substrates. Scanning electron microscopy

showed direct degradation of hyphae when *S. rolfssii* mycelium served as sole carbon source for the bacterium.

Crude chitinase of *S. marcescens* caused lysis of hyphal tips. In fact, it has already been shown that hyphal tips, or other specific regions, are sensitive to enzymatic degradation. For instance, Kritzman et al (13) showed that the active sites in the tips, septa, and branches of hyphae contain oligomers of β -glucan and GlcNac. We may, therefore, conclude that chitinase produced by the biocontrol agent attacks these sites, and probably causes a release of *S. rolfssii* β -glucanase, which, together with the chitinase, completely degrades the hyphae. Though there may be other mechanisms involved in the biocontrol ability of *S. marcescens*, it appears that lytic ability plays a role in control of the plant pathogenic fungus *S. rolfssii*.

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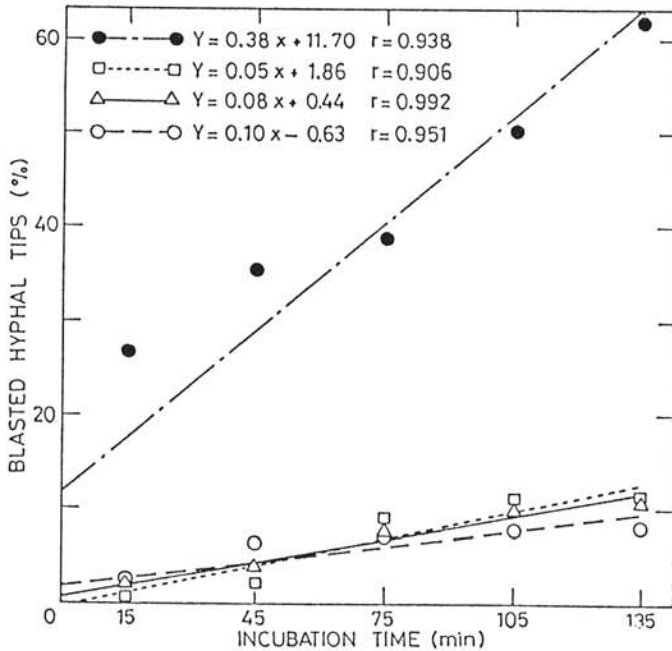


Fig. 4. Lysis of *Sclerotium rolfssii* hyphal tips by lytic activity of *Serratia marcescens*. Lines represent regression analysis of arc sin $\sqrt{x/100}$ (x = percentage of blasted hyphal tips). The edges of 2-day-old *S. rolfssii* cultures were treated with: *S. marcescens* enzyme filtrate (EF) (●-●-●), boiled EF (□-□-□), autoclaved EF (○-○-○), and distilled water (Δ-Δ-Δ).

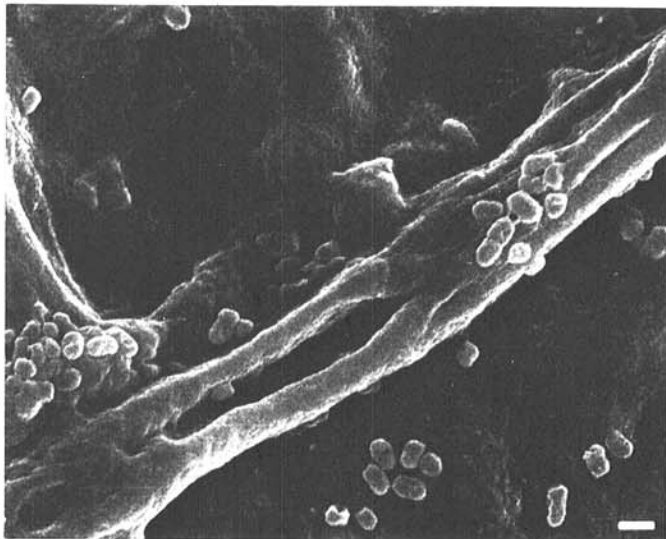


Fig. 5. Scanning electron micrograph showing partial degradation of *Sclerotium rolfssii* hyphae caused by *Serratia marcescens* and accumulation of bacterial cells around the host (bar = 1 μ m).

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