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ORIGINAL ARTICLE

Chitinolytic activity of endophytic *Streptomyces* and potential for biocontrol

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Abstract

Aims: Biological sources for the control of plant pathogenic fungi remain an important objective for sustainable agricultural practices. Actinomycetes are used extensively in the pharmaceutical industry and agriculture owing to their great diversity in enzyme production. In the present study, therefore, we evaluated chitinase production by endophytic actinomycetes and the potential of this for control of phytopathogenic fungi.
Methods and Results: Endophytic *Streptomyces* were grown on minimum medium supplemented with chitin, and chitinase production was quantified. The strains were screened for any activity towards phytopathogenic fungi and oomycetes by a dual-culture *in vitro* assay. The correlation between chitinase production and pathogen inhibition was calculated and further confirmed on *Colletotrichum sublineolum* cell walls by scanning electron microscopy.
Conclusions: This paper reports a genetic correlation between chitinase production and the biocontrol potential of endophytic actinomycetes in an antagonistic interaction with different phytopathogens, suggesting that this control could occur inside the host plant.
Significance and Impact of the Study: A genetic correlation between chitinase production and pathogen inhibition was demonstrated. Our results provide an enhanced understanding of endophytic *Streptomyces* and its potential as a biocontrol agent. The implications and applications of these data for biocontrol are discussed.

Introduction

Endophytic micro-organisms have received considerable attention for their potential as biocontrol agents of fungal plant pathogens. Varied enzyme production may result in new biochemical characteristics and in part be responsible for the inherent biodiversity of endophytic micro-organisms. Among the lytic enzymes evaluated as a source of biocontrol agents, chitinases have been studied largely because these enzymes are produced by a variety of endophytic micro-organisms (El-Tarabily and Sivasithamparam 2006). Many species of bacteria, fungi and plants produce chitinolytic enzymes, which can vary both within and among microbial species, depending on the number, types and positions of discrete binding and catalytic domains (Kobayashi *et al.* 2002). Chitinases are divided into three categories according to their enzymatic function: exochitinase, endochitinase and chitobiase, which constitute a complex of different degradation enzymes (Vyas and Deshpande 1989).

Bacterial chitinases have been widely demonstrated as inhibiting fungal growth and can therefore be effective in controlling plant-pathogenic fungal diseases (Ordentlich *et al.* 1988). One possible explanation for fungal inhibition is the action of chitinases in fungal cell walls, acting as plant protective agents (Inbar and Chet 1991). However, chitinases are not fully effective in all circumstances owing to different environmental conditions (Gohel *et al.* 2006). Endophytic actinomycetes have been cited as promising biocontrol agents, either acting directly on

fungal cell walls or initiating increased plant responses against disease. In addition, research indicates that both mechanisms operate to control fungal pathogens (Gupta *et al.* 1995; Coombs *et al.* 2004). In the present study, chitinase production by endophytic actinomycetes and their anti-fungal activities against phytopathogens was quantified. In addition, the correlation between chitinase production and fungal inhibition was evaluated.

Materials and methods

Micro-organism strains and growth conditions

Twenty-five strains of *Streptomyces* spp. cultures (Table 1) were previously isolated from citrus (23 strains) and soyabean (two strains) plants (Marcon 2000). Cultures were grown and maintained on solid starch medium (Küster and Williams 1964) at 28°C. The following phytopathogenic fungi were included in the study: *Phytophthora parasitica* and *Guignardia citricarpa* from *Citrus* sp. and *Rhizoctonia solani*, *Colletotrichum sublineolum*, *Pythium* sp. and *Fusarium oxysporum* from *Phaseolus vulgaris, Sorghum vulgare*, *Lactuca sativa* and *Saccharum* sp., respectively. These fungi were maintained on potato–dextrose agar (PDA) at room temperature. All isolates were maintained in the Culture Collection at the Laboratory of Microbial Genetics, Department of Genetics, ESALQ/USP, Piracicaba, SP, Brazil.

Table 1 Origin host and identification of endophytic *Streptomyces* spp

Strains	Host plant	Species
A6	*Citrus sinensis*	*Streptomyces somaliensis*
A11		*S. somaliensis*
A12		*S. somaliensis*
A15		*Streptomyces* sp.
A18		*Streptomyces* sp.
A20		*Streptomyces* sp.
A21		*Streptomyces cyaneus*
A22		*Streptomyces purpurascens*
A26		*Streptomyces* sp.
A28		*Streptomyces* sp.
A29		*Streptomyces griseus*
A30		*Streptomyces cyaneus*
A31		*Streptomyces purpurascens*
A32		*Streptomyces* sp.
A34		*S. griseus subsp. griseus*
B1		*Streptomyces* sp.
A1	*Citrus reticulata*	*Streptomyces* sp.
A8		*Streptomyces diastatochromogenes*
A10		*Streptomyces* sp.
A19		*Streptomyces bicolor*
A13		*Streptomyces* sp.
A23		*Streptomyces wadayamensis*
Arg	*Citrus limonia*	*S. purpurascens*
S29	*Glycine max*	*Streptomyces* sp.
S39		*Streptomyces* sp.

Isolation and quantification of extracellular chitinase

To generate extracellular chitinase, *Streptomyces* were grown in M9 salt broth supplemented with 0·5% insoluble chitin. After 5 days of growth at 28°C, the cells were pelleted by centrifugation (10 000 ***g*** for 5 min) and the culture filtrates were maintained at 4°C for further analysis. Chitinase activity in culture filtrates was assayed using CM-Chitin RBV in 50 mmol l^{-1} Tris-HCl buffer (pH 7·5) at 45°C. The values were standardized based on protein concentration (Bradford 1976). Chitin degradation was quantified in a spectrophotometer (Ultrospec 3000; Pharmacia/Biotech) for CM-chitin absorbance at 550 nm. The enzyme activity was measured in absorbance per millilitre of substrate reaction per hour (Guzzo *et al.* 1999).

Evaluation of antagonistic bacteria

Streptomyces spp. were evaluated for activity towards *P. parasitica, G. citricarpa, R. solani*, *C. sublineolum*, *Pythium* sp. and *F. oxysporum* by a dual-culture *in vitro* assay onto PDA plates. The diameter of the inhibition areas was measured after 5 days of incubation at 28°C. All the strains were tested in four independent replicates. Only *Streptomyces* isolates that showed antagonistic activity towards fungi and high chitinase production were evaluated for their ability to degrade fungal cell walls by scanning electron microscopy (SEM; DSM940A, Zeiss, Germany).

Scanning electron microscopy

SEM was employed to evaluate the effects of *Streptomyces* spp. (strains A8 and A1) culture filtrates on the fungal cell walls of *C. sublineolum* and *Pythium* sp. The fungi were cultivated in PD broth and the mycelium was treated at 28°C with crude extract from A8 or A1 isolates in chloride solution (0·85%). After 3 h, the mycelium was fixed overnight in cacodylic acid (2 mol l^{-1}) and glutaraldehyde (8%) solutions and washed twice in cacodylic acid solution (1 mol l^{-1}). The samples were then dehydrated in a graded acetone series, critical-point dried with carbon dioxide, coated with gold–palladium and observed by SEM.

Statistical analysis

All data (chitinase production and antagonistic isolates) were analysed for significance ($P < 0{\cdot}01$) using the SAS (version 6·11) software package. A completely randomized

analysis was employed because the inhibition pathogen data was transformed to ($\sqrt{x}$ + 1) by variance stabilization. Statistical differences between treatments were determined at a 1% level of significance. For covariance and correlation estimates between these data, an analysis of variance (ANOVA) was applied. The correlation between anti-fungal and chitinase activities was assessed by genotypic (r_G) and phenotypic correlation (r_P) coefficients ($r_{x,y}$). A significant correlation coefficient and statistical differences between the pathogenic fungi were determined at the 5% level of significance (Zar 1984).

Table 3 Estimates between chitinase production and anti-fungal activity of phenotypic (r_P) and genotypic (r_G) correlation coefficients

Phytopathogenic fungi	r_P	r_G
Colletotrichum sublineolum	0·790833*	0·759939*
Guignardia citricarpa	0·6513*	0·622149*
Rhizoctonia solani	0·706423*	0·650574*
Fusarium oxysporum	0·659171*	0·606209*
Pythium sp.	0·43946	0·414257
Phytophthora parasitica	0·028441	0·022076

The data marked with * are the correlations that are statistically significant ($P < 0·05$).

Results

Chitinolytic and antimicrobial activities

The endophytic *Streptomyces* strains were screened for chitinase production and inhibition of fungi and oomycete phytopathogens. Most strains produced a high concentration of chitinase and inhibited the phytopathogens. However, the inhibition of phytopathogens within each strain was completely dependent on the species studied (Table 2). *Streptomyces* spp. generally induced larger fungal growth inhibition zones for *G. citricarpa, R. solani*, *C. sublineolum* and *F. oxysporum* than for oomycetes *P. parasitica* and *Pythium* sp. (Table 2).

Table 2 Chitinolytic and antagonistic activities of *Streptomyces* spp. strains

		Anti-fungal activities (diameter of zone of inhibited fungal growth/diameter of bacterial colonies) of					
Strains	Chitinase activity index*	*Colletotrichum sublineolum*	*Guignardia citricarpa*	*Rhizoctonia solani*	*Fusarium oxysporum*	*Phytophthora parasitica*	*Pythium* sp.
A1	0·77 c	0·00 f	0 e	0 d	1·87 bcde	0 g	0·00 e
A6	2·03 abc	3·01 bcd	2·06 a	2·06 c	2·22 abcde	1 f	0·00 e
A8	5·58 a	3·77 abc	1·96 ab	3·20 a	2·20 abcde	1·07 f	1·02 cd
A10	4·10 abc	4·43 ab	2·25 a	2·30 bc	2·18 abcde	1·46 cdef	1·10 cd
A11	4·33 abc	0·00 f	1 d	0 d	0·00 f	1·75 bcd	0·00 e
A12	3·69 abc	0·00 f	1·19 cd	0 d	0·00 f	0 g	0·00 e
A13	6·22 a	3·30 bcd	1·73 abcd	2·27 bc	2·90 ab	1·19 ef	1·00 cd
A15	5·60 a	5·51 a	1·99 ab	2·07 c	0·61 abcd	1·69 bcde	1·13 bcd
A18	5·54 a	2·89 bcd	2·21 a	1·79 c	2·95 ab	1·07 f	1·05 cd
A19	0·67 c	1·57 def	1·24 bcd	0 d	0·00 f	3·50 a	3·16 a
A20	4·07 abc	3·77 abc	2·15 a	0 d	2·74 abcd	2·13 b	1·28 bc
A21	4·39 abc	0·00 f	2·33 a	0 d	0·00 f	1·99 bc	0·00 e
A22	0·62 c	0·00 f	1·07 d	0 d	0·00 f	1 f	0·00 e
A23	5·40 a	4·72 ab	1·90 abc	1·71 c	2·38 abcde	1·39 def	1·40 b
A26	5·39 a	3·29 bcd	2·25 a	1·97 c	1·75 bcde	1·19 ef	0·93 d
A28	5·42 a	4·20 abc	1·93 cd	2·27 bc	2·82 abc	1·05 f	1·18 bcd
A29	5·83 a	2·31 cde	2·06 a	2·07 c	1·63 cde	1·13 f	1·07 cd
A30	3·60 abc	0·00 f	2·14 a	0 d	0·00 f	2·07 b	0·00 e
A31	3·40 abc	0·00 f	1·18 cd	0 d	0·00 f	1·30 def	0·00 e
A32	5·05 ab	3·83 abc	2·22 a	2·11 c	1·38 e	1·26 def	1·13 bcd
A34	6·37 a	4·30 ab	2·22 a	2·13 c	3·20 a	1·49 cdef	1·02 cd
B1	5·47 a	3·33 cde	2·00 a	1·72 c	2·49 abcde	0 g	0·00 e
S29	1·13 bc	1·47 def	2·13 a	2·97 ab	2·17 abcde	2·16 b	0·00 e
S39	3·61 abc	0·50 ef	2·13 a	0 d	1·57 de	0·00 g	0·00 e
ARG	5·36 a	3·83 abc	2·28 a	3·06 a	2·73 abcd	1·37 def	1·16 bcd

Values with the same letter within a column are not significantly ($P > 0·01$) different according to the SAS System (release 6·11). The results are means of four replicates for each strain and fungus.

*The chitinolytic activity was measured in absorbance per millilitre of substrate × reaction per hour.

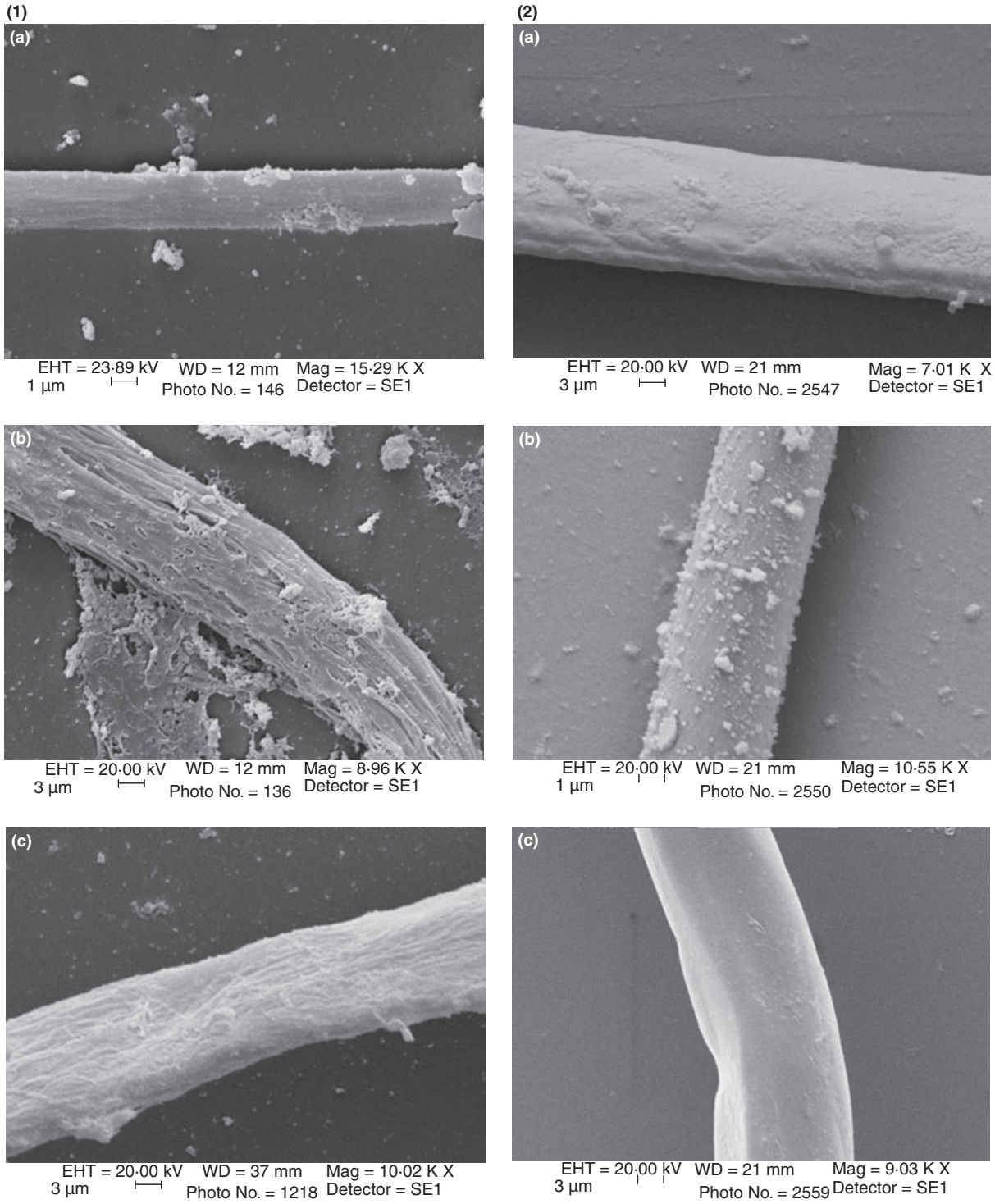

Figure 1 Scanning electronic microscopic analysis of: (1) *Colletotrichum sublineolum* hyphae and (2) *Pythium* sp. hyphae. Control: fungi hyphae on saline solution (a). Chitinase action: hyphae fungi, after incubation at 28°C for 3 h in crude extract chitinolytic of A8 strain (b) and A1 strain (c). Bars indicate 10 μm.

A positive genetic and phenotypic correlation was indicated between the relative chitinolytic and antagonistic activity against *C. sublineolum, R. solani, G. citricarpa* and *F. oxysporum*. However, the inhibition of *P. parasitica* and *Pythium* sp. was not correlated to chitinolytic activity (Table 3).

Effect of culture filtrate on microbial cell walls

The effect of high and low chitinolytic A8 and A1 strains, respectively, on *C. sublineolum* and *Pythium* sp. mycelium was evaluated using SEM. Observations revealed that the *C. sublineolum* cell wall was degraded by the chitinolytic strain A8 but not by A1, a low-chitinolytic strain (Fig. 1a). The *C. sublineolum* hyphae surface-treated with A8 culture filtrate contained many holes, possibly corresponding to lysis zones. However, the hyphal surfaces of both *C. sublineolum* and *Pythium* sp. treated with A8 culture filtrate exhibited a slightly roughened surface, indicating little to no effect of hydrolytic enzymes on these structures (Fig. 1). Furthermore, *C. sublineolum* and *Pythium* sp. hyphae treated with saline solution exhibited a smooth surface.

Discussion

Endophytes are micro-organisms that inhabit plant interior tissues. These micro-organisms cause no harm to the host and do not develop external structures, which exclude nodulating bacteria and mycorrhizal fungi as endophytic micro-organisms (Azevedo and Araújo 2007). Owing to the innocuous nature of these organisms, delivery systems to introduce endophytic bacteria, such as *Streptomyces* spp., into plants have been developed, and the potential for endophytes as biocontrol agents has been explored (Bhattacharya *et al.* 2007). In a recent study, a number of endophytic actinomycetes suppressed wheat fungal pathogens, including *R. solani*, *Pythium* sp. and *Gaeumannomyces graminis* var. *tritici*, both *in vitro* and in plants, indicating their potential use as biocontrol agents (Bendt *et al.* 2001). Shekhar *et al.* (2006) purified a bioactive compound from endophytic *Streptomyces violaceusniger* that showed a strong antagonism towards various wood-rotting fungi, and chitinase enzymes were associated with this inhibition. In general, the higher chitinase activity was correlated with higher fungal inhibition. For this reason, chitinolytic *Streptomyces* strains are a likely choice as potential biological control agents.

In a similar study, Aktuganov *et al.* (2003) evaluated 19 chitinolytic *Bacillus* strains. The enzymatic preparations of most of the 19 strains inhibited *Helminthosporium sativum* growth; however, a correlation between enzymatic activity and growth inhibition was not detected. In the present study, we evaluated the antagonistic effects of endophytic and chitinolytic *Streptomyces* towards fungi and oomycetes. A correlation between chitinolytic and antagonistic activities was observed for fungi but not oomycetes, which possess cellulose as their main cell wall compound. Chitinolytic activity has been implicated in the biocontrol activity of several bacteria, including *Streptomyces* spp. However, for many biocontrol systems, a direct statistical correlation for the role of chitinase is lacking.

In the present study, the chitinolytic strains showed high inhibition levels against fungi, and the fungal hyphae exhibited a degraded appearance after chitinolytic A8 strain culture treatment. These data indicated an inhibitory role of chitinase to plant pathogenic fungi, but not to oomycetes. Similar results were observed with crude and purified enzymes from *Streptomyces aureofaciens*, resulting in cell wall lysis in many phytopathogenic fungi (Taechowisan *et al.* 2003).

This study provides a quantitative assessment of *Streptomyces* chitinolytic and antimicrobial activities. In addition, an estimative of the genetic parameters associated with these processes provides a foundation for micro-organism screening programmes to search for fungal antagonistic agents. However, it should be noted that chitinase activity represents one component of a complex suite of phenotypic characteristics. The virulence of a given isolate against a host population is dictated by specific environmental conditions. Furthermore, in mature plants the niche occupied by the endophytes becomes more stable and uniform, resulting in selective pressures that may favour specific genotypes within each local microbial population (Lacava *et al.* 2004). However, the plant cell wall lacks chitin and the presence of chitinolytic endophytes may indicate that genotypic selection inside the host plant might favour alleles with the ability to control fungal pathogens.

The results of this study led us to conclude that chitinase produced by endophytic *Streptomyces* has the potential for control of plant pathogenic fungi. However, a further evaluation of the disease control effectiveness of these strains and the design of a biocontrol formulation and application must be conducted under field conditions.

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