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SHORT COMMUNICATION

Extracellular enzyme production and fungal mycelia degradation of antagonistic *Streptomyces* induced by fungal mycelia preparation of cucurbit plant pathogens

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Abstract *Streptomyces* are beneficial soil microorganisms and potential candidates for biocontrol agents against soilborne pathogenic fungi of cucurbit plants. Extracellular enzymes such as cellulase, chitinase and glucanase produced by *Streptomyces* are important components of actinomycete-fungus antagonism. This study aimed to investigate the influence on extracellular enzymes production and fungal mycelia degradation by antagonistic *Streptomyces* of mycelia preparation of pathogenic fungi (MPPF) of cucurbit plants. The results showed that the antagonistic *Streptomyces* displayed high extracellular enzyme activities to varying degrees when MPPF was used as the sole carbon source. The MPPF from *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *niveum* and *Alternaria tenuissima* were the most effective carbon sources in enhancing the cellulase activity of *Streptomyces globisporus* C7, *Streptomyces globisporus* subsp. *globisporus* C28 and *Streptomyces kanamyceticus* C49, respectively. *S. globisporus* subsp. *globisporus* C28, *Streptomyces pactum* A12 and *S. kanamyceticus* C49 cultured in the medium containing MPPF from *Fusarium equiseti* showed the highest chitinase activity (12.35, 12.50 and 15.06 U, respectively) of all the MPPF treatments. Glucanase activity of *Streptomyces carnosus* A11 was enhanced greatly (9.26 U) when MPPF from *A. tenuissima* was used as the sole carbon source. A hyphal intertwining and degradation phenomenon was observed when the antagonistic *Streptomyces* came across the pathogenic fungal mycelia, which was due to a synergistic effect of the extracellular enzymes produced by the antagonistic *Streptomyces*.

Keywords Extracellular enzyme · Mycelia preparation of pathogenic fungi · *Streptomyces* · Cucurbit plant pathogen · Fungal mycelia degradation

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Soilborne diseases are the most probable cause of continuous cropping obstacles in cucurbit plants such as melon, watermelon and cucumber. Some antagonistic *Streptomyces* suggest the potential for biocontrol in the management of such soilborne diseases (El-Tarabily et al. 2009; Zhao et al. 2012). Besides antibiotic production, another biocontrol mechanism of the antagonistic *Streptomyces* involves the production of extracellular enzymes (Quecine et al. 2008; Rabeeth et al. 2011). Extracellular enzymes are produced by various microorganisms as hydrolytic enzymes, attack the structural components of cell walls of most fungi (Fayad et al. 2001; Hayat et al. 2010) and play an important role in fungal mycelia degradation. Previous research has concentrated mainly on the induction of extracellular enzymes produced by fungi (Shi et al. 2008; Manczinger et al. 2001). For example, the chitinase activity of *Trichoderma* sp. could be enhanced by growth in medium containing colloidal chitin or a cell wall preparation of *Rhizoctonia solani* and *Botrytis cinerea* (Xu and Liu 2002). The synthesis of glucanase by *Chaetomium* spp. could be induced by a cell wall preparation from *Magnaporthe grisea*, *Rhizoctonia solani* and *Fusarium oxysporum* (Chen et al. 2010). Little research has focused on the synchronous induction of extracellular enzymes produced by antagonistic *Streptomyces*. In this study, we investigated the influence of MPPF on extracellular enzyme production and fungal mycelia degradation by antagonistic *Streptomyces*, so as to understand the biocontrol

Table 1 Cellulase activities (U) of *Streptomyces* grown in media containing mycelia preparation of pathogenic fungi (MPPF) or starch as the sole carbon source. Values are the means (± SE) of three replicates conducted under the same experiment conditions. Different letters within the same column indicate significant differences according to Duncan's new multiple range test ($P<0.05$)

Carbon source		*Streptomyces* strain number					
		C7	C28	A11	A12	C49	D153
Starch		8.51±0.10 b	16.35±0.16 a	8.00±0.21 ab	9.42±0.26 a	9.38±0.41 ab	7.95±0.60 bcd
MPPF	*Alternaria tenuissima*	7.13±0.45 d	7.40±0.12 d	7.40±0.37 bc	8.12±0.52 b	9.65±0.44 a	9.05±0.26 a
	Botryosphaeria dothidea	8.10±0.34 bc	8.92±0.24 bc	8.39±0.11 a	7.08±0.31 c	7.60±0.10 c	7.81±0.77 bcd
	Fusarium equiseti	7.51±0.23 cd	8.30±0.28 c	7.77±0.30 ab	8.22±0.26 b	8.79±0.42 b	7.51±0.46 cd
	Fusarium proliferatum	9.48±0.76 a	8.42±0.85 c	7.36±0.31 bc	9.25±0.24 a	7.03±0.38 c	8.67±0.24 ab
	Fusarium oxysporum f. sp. *niveum*	8.65±0.50 b	9.62±0.27 b	7.06±0.49 c	8.88±0.35 a	7.00±0.20 c	8.10±0.61 bc
	Fusarium oxysporum f. sp. *cucumerinum*	7.95±0.09 bc	9.13±0.68 bc	7.83±0.54 ab	7.38±0.05 c	8.82±0.75 b	7.07±0.20 d

mechanism of *Streptomyces* against soilborne cucurbit plant pathogens.

Six pathogenic fungi of cucurbit plants, i.e., *Alternaria tenuissima*, *Botryosphaeria dothidea*, *Fusarium equiseti*, *Fusarium proliferatum*, *Fusarium oxysporum* f. sp. *niveum* and *Fusarium oxysporum* f. sp. *cucumerinum* were used. The *Streptomyces* were isolated from the special habitat soil of the Qinghai-Tibet Plateau of China on Gause I medium. During a screening program focused on their antagonism against pathogenic fungi, six *Streptomyces* displayed an obvious inhibition effect on soilborne cucurbit plant pathogens (Sun et al. 2009; Zhao et al. 2011). These six *Streptomyces*, i.e., C7, C28, A11, A12, C49 and D153, were subsequently identified as *Streptomyces globisporus*, *Streptomyces globisporus* subsp. *globisporus*, *Streptomyces carnosus*, *Streptomyces pactum*, *Streptomyces kanamyceticus* and *Streptomyces cyaneofuscatus*, respectively, according to their morphological, physiological and biochemical characteristics and 16 S rDNA sequence analysis.

The pathogenic fungi were grown in 250 mL Erlenmeyer flasks containing 100 mL potato dextrose broth and incubated at 25 °C on a rotary shaker (150 rpm) for 5 days. Fungal mycelia were harvested with four-fold cotton gauzes, washed twice to remove the growth medium and steam sterilized at 121 °C for 30 min. The MPPF was obtained after oven-drying fungal mycelia at 40 °C for 12 h and grinding to a powder. For extracellular enzyme induction, 1 % (w/v) MPPF was added to Gause I medium and used as the sole carbon source instead of starch. *Streptomyces* were grown in 250 mL Erlenmeyer flasks containing 100 mL Gause I broth (1 % MPPF instead of starch) and cultured at 28 °C on a rotary shaker (150 rpm) for 7 days. The contents of the flasks were collected and filtered through sterile 0.22 μm pore-size Millipore filters (Phae et al. 1990). The filtrates were then used as crude enzyme and kept at 4 °C for detection of enzyme activity.

Cellulase activity was assayed by the amount of glucose generated from sodium carboxymethyl cellulose as described by Cheng and Xue (2000). A unit of enzyme activity was defined as the amount of glucose produced per minute by 1 mL crude enzyme at 40 °C. Chitinase activity was measured by the amount of N-acetyl glucosamine generated from colloidal chitin (Gupta et al. 1995). A unit of enzyme activity was

Table 2 Chitinase activities (U) of *Streptomyces* grown in media containing MPPF or starch as the sole carbon source. Values are the means (± SE) of three replicates conducted under the same experiment conditions. Different letters within the same column indicate significant differences according to Duncan's new multiple range test ($P<0.05$)

Carbon source		*Streptomyces* strain number					
		C7	C28	A11	A12	C49	D153
Starch		16.90±0.44 a	12.90±0.32 a	8.20±0.10 bc	13.87±0.26 a	8.40±0.65 c	22.96±0.22 a
MPPF	*Alternaria tenuissima*	10.74±0.06 bc	8.26±0.40 d	9.47±0.33 a	7.85±0.47 f	8.12±0.26 c	13.23±0.22 b
	Botryosphaeria dothidea	7.80±0.43 e	7.48±0.34 d	8.71±0.20 b	9.87±0.21 d	8.15±0.77 c	8.35±0.35 e
	Fusarium equiseti	11.11±0.15 b	12.35±0.46 a	8.05±0.28 c	12.50±0.37 b	15.06±0.27 a	13.44±0.11 b
	Fusarium proliferatum	9.08±0.49 d	8.22±0.64 d	9.48±0.33 a	10.87±0.30 c	11.32±0.18 b	8.85±0.22 e
	Fusarium oxysporum f. sp. *niveum*	10.69±0.39 bc	11.00±0.51 b	9.26±0.45 a	9.33±0.72 de	8.92±0.11 c	12.49±0.10 c
	Fusarium oxysporum f. sp. *cucumerinum*	9.90±0.90 cd	10.08±0.62 c	8.21±0.19 bc	8.74±0.08 e	9.00±0.88 c	11.15±0.67 d

Table 3 Glucanase activities (U) of *Streptomyces* grown in media containing MPPF or starch as the sole carbon source. Values are the means (± SE) of three replicates conducted under the same experiment conditions. Different letters within the same column indicate significant differences according to Duncan's new multiple range test ($P<0.05$)

Carbon source		*Streptomyces* strain number					
		C7	C28	A11	A12	C49	D153
Starch		8.57±0.25 a	21.90±0.64 a	12.78±0.45 a	16.43±0.50 a	15.59±0.21 a	8.53±0.13 a
MPPF	*Alternaria tenuissima*	0 d	0 c	9.26±0.37 b	0 c	0 b	8.46±0.08 a
	Botryosphaeria dothidea	7.36±0.26 c	0 c	0 c	0 c	0 b	7.71±0.60 b
	Fusarium equiseti	0 d	8.45±0.41 b	0 c	0 c	0 b	0 c
	Fusarium proliferatum	8.25±0.02 b	0 c	0 c	0 c	0 b	7.94±0.33 ab
	Fusarium oxysporum f. sp. *niveum*	0 d	0 c	0 c	7.05±1.92 b	0 b	8.37±0.70 ab
	Fusarium oxysporum f. sp. *cucumerinum*	8.17±0.11 b	0 c	0 c	7.87±0.06 b	0 b	0 c

defined as the amount of N-acetyl glucosamine produced per minute by 1 mL crude enzyme at 37 °C. Glucanase activity was determined by measuring the amount of glucose released from laminarin (Chan and Tian 2005). One unit of glucanase activity was defined as the amount of glucose produced per minute by 1 mL crude enzyme at 40 °C. All measurements of enzymatic activities were performed in triplicate for each sample. Background levels of glucose were determined with crude enzymes after boiling at 100 °C for 10 min.

Degradation of fungal mycelia by the antagonistic *Streptomyces* was observed according to the method described by Cheng and Xue (2000). A disinfected tiny spade was used to dig out two parallel grooves (0.5 cm width) on a PDA plate. *Streptomyces* was inoculated on one edge of the groove while pathogenic fungus was inoculated on the other, and sterile cover glasses were used to gently cover the groove face. After incubating at 28 °C for 5 days, the cover glasses were removed with sterile tweezers with the side of mycelia facing up. Microscopical examination was conducted by observing the cover glasses under a microscope.

The MPPF of cucurbit plants displayed a synchronous induction effect on the production of *Streptomyces* extracellular enzymes. *S. kanamyceticus* C49 showed the highest cellulase activity (9.65 U) of all the MPPF treatments when MPPF from *A. tenuissima* was used as the sole carbon source. *S. globisporus* subsp. *globisporus* C28 cultured in medium containing MPPF

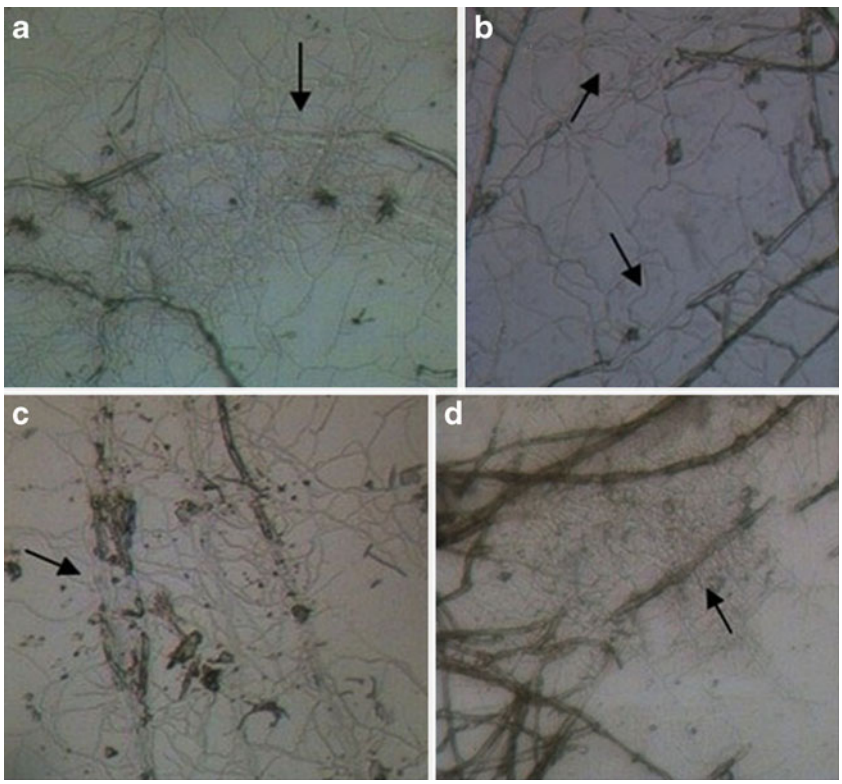

Fig. 1a–d Microscopic examination of fungal mycelia degradation of cucurbit plant pathogens by antagonistic *Streptomyces* (magnification 40×). **a** *Streptomyces* A12 against *Alternaria tenuissima*; **b** *Streptomyces* C7 against *Botryosphaeria dothidea*; **c** *Streptomyces* A12 against *B. dothidea*; **d** *Streptomyces* A11 against *A. tenuissima*

from *F. oxysporum* f. sp. *niveum* also displayed higher cellulase activity (9.62 U) compared with other MPPF treatments (Table 1). The chitinase activity of *S. kanamyceticus* C49 reached a high of 15.06 U when cultured in medium using MPPF from *F. equiseti* as the sole carbon source ($P<0.05$). *S. carnosus* A11 cultured in medium containing MPPF from *F. proliferatum* also displayed higher chitinase activity compared with other treatments. Conversely, the other four *Streptomyces* strains showed higher chitinase activities when starch was used as the sole carbon source (Table 2). High glucanase activities were obtained when *Streptomyces* were cultured in medium with 1 % starch as the sole carbon source. The glucanase activity of *S. carnosus* A11 cultured in medium containing MPPF from *A. tenuissima* was 9.26 U—a little lower than that cultured in medium containing starch. The glucanase activities of *S. cyaneofuscatus* D153 varied from 7.71 to 8.46 U when cultured in medium containing MPPFs from *A. tenuissima*, *B. dothidea*, *F. proliferatum* and *F. oxysporum* f. sp. *niveum* (Table 3). Under a microscope, an abnormal hyphal intertwining and degradation phenomenon was observed when *Streptomyces* came across the fungal mycelia of cucurbit plant pathogens (Fig. 1).

Most pathogenic fungi have cell walls composed of complex polymers of β-1, 3- and β-1, 6- glucans, where chitin acts as a structural backbone arranged in regularly ordered layers, and glucan as a filling material arranged in an amorphous manner (Cheng et al. 2009; Smits et al. 2001). Thus, breakdown of the fungal cell wall requires the participation of different hydrolytic enzymes (Marcello et al. 2010). In this study, the antagonistic *Streptomyces* showed obvious extracellular enzymes activities to varying degrees when MPPF from soilborne cucurbit plant pathogens was used as the sole carbon source. These enzymes worked synergistically in the process of fungal mycelia degradation.

The mechanisms employed by *Streptomyces* in the biocontrol of pathogenic fungi of cucurbit plants include mainly competition for space or nutrients, production of antifungal metabolites, and secretion of hydrolytic enzymes such as chitinases and glucanases (Neeraja et al. 2010). This study demonstrated the contact and degradation mechanism of antagonistic *Streptomyces* against the cucurbit plant pathogens in terms of enzymolysis. The induction effect of MPPF on extracellular enzymes provided definite proof of the biocontrol mechanisms of the antagonistic *Streptomyces* against soilborne cucurbit plant pathogens.

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