Assessment of Production of Extracellular Enzymes by *Trichoderma* spp. for Control of Soybean Root Rot Pathogens (*Fusarium oxysporum*, *Rhizoctonia solani*)*

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Abstract The role of extracellular enzymes by *Trichoderma* MM35 for control of soybean root rot pathogens(*Fusarium oxysporum*, *Rhizoctonia solani*) was assessed *in vitro* and *in vivo*. Detective levels of hydrolytic extracellular enzymes were recorded by *Trichoderma* MM35 using dried *F. oxysporum* mycelium as C source in *vitro* or fresh *F. oxysporum* mycelium or fresh *R. solani* mycelium *in vivo* was found that there were significant increases in chitinase activities by *Trichoderma* MM35 in soil with inoculation of *F. oxysporum*. Soil infested with *Trichoderma* MM35 had significantly elevated chitinase and β－1, 3－glucanase activities in presence of *R. solani* as compared to *R. solani* control.

Key words *Trichoderma* spp.; Root rot of soybean; Mechanism; Extracellular enzyme, Chitinase; β－1, 3－glucanase

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Trichoderma spp. are among the most common saprophytic fungi in the rhizosphere. Of these, some isolates of *Trichoderma* spp. have been proven to be effective as biocontrol agents against a wide range of important airborne and soilborne plant pathogens[1]. The proposed mechanisms by which *Trichoderma* strains antagonize pathogenic populations include the production of antibiotics and/or fungal cell wall-degrading enzymes, as well as competition for nutrients in the rhizosphere[2]. The plant pathogens (*F. oxysporum*, *R. solani*) causes soybean root rot which are major constraints to production in many soybean-growing regions in Heilongjiang province of China. The disease is protected by using fungicides. Furthermore, fungi also can cause many diseases in hundreds of other plant species. Out of 128 *Trichoderma* isolates, one strain *Trichoderma* MM35 was screened with good biocontrol against soybean root rot pathogens. With a view to establish that the mechanism in the process of antagonism of *F. oxysporum* or *R. solani* by the newly isolated strain involves the release of hydrolytic enzymes by the latter, the objectives of this study were to determine the production of chitinase and β-1, 3-glucanase *in vitro* and *in vivo*, and to assess the role

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of these enzymes in antagonism.

1 Materials and Methods

1.1 Microorganisms and cultivation

Trichoderma MM35 (isolated from soybean rotation plots of Hailun ecology research of Chinese Academy of Sciences and ever tested with biocontrol ability, data shown in table 1 and soybean root rot pathogens(*F. oxysporum*, *R. solani*).

PDA, synthetic medium (in grams per liter): NH_4NO_3 5 g, KH_2PO_4 10 g, NaCl 1 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, H_2O 1000 mL[pH 6.5], wheat bran medium(wheat bran: sawdust 15 g: 15 g, moistened with 30 mL water and sterilized for 15 min at 121 ℃).

1.2 Antagonism of *Trichoderma* MM35 against *F. oxysporum* or *R. solani* in *vitro*

Antagonism of *Trichoderma* MM35 against *F. oxysporum* or *R. solani in vitro* was observed by the method proposed by Elad et al[3].

1.3 Preparation of dried mycelium

Dried mycelium of *F. oxysporum* was prepared by the method proposed by El-Katatny[4].

1.4 Preparation of the inoculum used for infesting the potting mixture

Four discs(5 mm diameter) were cut from the edge of growing *Trichoderma* MM35 colony on PDA and added to the 50 mL flask containing 20 g wheat bran medium. The flasks were incubated at 25 ℃ in darkness for 7 days. 100g kafirin was added into an Erlenmeyer flask (250 mL), moistened 12 h, sterilized at 121 ℃ for 30 min on each of two consecutive days. Five disks from the margin of a colony of *F. oxysporum* growing on PDA were transferred to the flask. The flask was incubated for 10 days. The same procedure was followed for *R. solani*. All of the inoculums were stored at 4 ℃ for used.

1.5 Conditions for enzyme production by *Trichoderma* MM35

Four discs of 5mm diameter (cut from the edge of actively *Trichoderma* MM35 colony growing on PDA) were inoculated in 500 mL flask with 200 mL synthetic medium supplemented with the mycelium of *F. oxysporum* (0.5g/L), incubated at 150 rpm on a rotary shaker for 5 days at 28 ℃. 10ml cultures were sampled after inoculation at 24 h, 48 h, 72 h, 96 h, 120 h, then centrifuged at 4 ℃for 20 min at 10000 g and the clear supernatants were stored at −20 ℃until assayed. The experiment was replicated twice, five replicates for each treatment.

1.6 Preparation and inoculation of the potting mix

Potting medium was prepared with 300g sterilized soil and infested with *F. oxysporum* inoculum or *R. solani* inoculum at a rate of 5%, or *Trichoderma* MM35 inoculum at a rate of 2.5%. The experimental design included the following treatments:(ⅰ) no *Trichoderma* MM35 or *F. oxysporum* or *R. solani*, (ⅱ) *F. oxysporum* but no *Trichoderma* MM35, (ⅲ) *R. solani* but no *Trichoderma* MM35, (ⅳ) *F. oxysporum* and *Trichoderma* MM35, and (ⅴ) R. solani and *Trichoderma* MM35. Mixing was accomplished by rotation in inflated plastic bags. Each combination was placed in 18×12×6.5 cm plots. 5 g of substrate was sampled for each treatment 6 to 16 days after inoculation. Soil samples were placed in plastic tubes and 10 mL sterile potassium phosphate (pH6.4) were added, gently stirred for 4 hours, and then centrifuged at 8000 g for 20min at 4 ℃. The supernatants were stored at −20 ℃ until assayed. The experiment was conducted twice. Each treatment was replicated five times.

1.7 Enzyme activity assays

The activities of chitinase and β-1,3-glucanase were assayed using the colorimetric method proposed by Molano et al.[4] and expressed in nanmol/min/mL or nanmol/min/g and μmol/min/mL or μmol/min/g, respectively.

2 Results

Obvious parasitism was observed in interaction of *Trichoderma* MM35 with *R. solani* by microscopy (Fig. 1), however, there was no parasit

ism in interaction of *Trichoderma* MM35 with *F. oxysporum* (*data not show*).

Detective levels of hydrolytic extracellular enzymes were recorded by *Trichoderma* MM35 using dried *F. oxysporum* mycelium as C-source *in vitro*. The production of hydrolytic extracellular enzymes by *Trichoderma* MM35 was time dependent. The significant elevation in the production of β-1, 3 glucanases was after 24 h, whilst that of chitinase appeared to significantly increase after 72 h(Fig. 2, Fig. 3).

Table 1　The result of greenhouse test (the first time)

Treatment [a]	Disease incidence (%)	Disease index	Relative control effect(%)
CK(*F. oxysporum*)	86.95	40.86	–
CK(Carbendazim)	24.13	8.96	78.07
MM35	59.37	13.75	66.34

(the second time)

Treatment[a]	Disease incidence (%)	Disease index	Relative control effect(%)
CK (*F. oxysporum*)[a]	61.53	22.30	–
CK (Carbendazim)[b]	34.48	6.89	69.1
MM35	51.85	16.29	26.95

a CK (*F. oxysporum*) control treated with *F. oxysporum*

CK (Carbendazim) control treated with *F. oxysporum* and Carbendazim MM35 treatment with *Trichoderma* MM35 and *F. oxysporum*

Detective levels of hydrolytic extracellular enzymes were also recorded by *Trichoderma* MM35 in soil in presence of fresh *F. oxysporum* mycelium or fresh *R. solani* mycelium *in situ* (table 2, table 3). There were significant increases in chitinase activities at day 4, day 9 and day 16 by *Trichoderma* MM35 in soil with inoculation of *F. oxysporum* as compared to *F. oxysporum* control (table 2). Soil infested with *Trichoderma* MM35 had significantly elevated chitinase activities at day 4 and day 12, and β-1, 3 glucanase activities at day 4, day 9, day 12 and day 16 in presence of *R. solani* as compared to *R. solani* control (table 2, table 3). Significant elevations in chitinase, β-1, 3 glucanase activities were also recorded in soil infested with *Trichoderma* MM35 only in comparison to *F. oxysporum* or *R. solani* control at day 4(table 2, table 3).

Table 2　Effect of *Trichoderma*, *F. oxysporum* and *R. solani* on the activity of chitinase in the soil

Soil-Treatment	4 day	9 day	12 day	16 day
T+*F*[a]	22.84±4.79ab[b]	46.98±27.99a	13.84±5.87b	43.33±12.98a
T+*R*	28.31±14.91a	9.52±4.00b	32.78±13.55a	9.52±8.20b
T	28.72±18.55a	15.28±3.82b	2.75±0.81b	49.82±31.65a
F	3.36±1.79c	5.46±1.87b	11.14±4.95b	11.43±4.01b
R	8.43±3.97bc	1.74±0.17b	1.77±0.15b	1.84±0.10b

a *T*+*F*= *Trichoderma* MM35 and *F. oxysporum*; *T*+*R* = *Trichoderma* MM35 and *R. solani*; *T* = *Trichoderma* MM35; F = F. *oxysporum*; *R*= *R. solani*; the activity of chitinase expressed as nanmol/min/g

b the means and standard error are obtained from five replicates, values followed by the same letter by column were not significantly different by Duncan's test(a=0.05)

Table 3　Effect of *Trichoderma*, *F. oxysporum* and *R. solani* on the activity of β-1, 3 glucanase in the soil

Soil-Treatment	4 day	9 day	12 day	16 day
T+*F*[a]	0.600±0.119 b[b]	ND[c]	0.91±0.37 b	1.27±0.22b
T+*R*	19.697±3.604a	20.91±0.14a	10.77±2.78 a	14.85±4.38a
T	19.692±3.604a	ND	0.06±0.11b	ND
F	0.004±0.005 b	ND	0.28±0.01b	ND
R	0.003±0.005 b	ND	ND	ND

a *T*+*F*= *Trichoderma* MM35 and *F. oxysporum*; *T*+*R* = *Trichoderma* MM35 and *R. solani*; *T* = *Trichoderma* MM35; F = F. *oxysporum*; *R*= *R. solani*; the activity of β-1, 3-glucanase expressed as umol/min/g

b Means followed by the same letter by column were not significantly different by Duncan's test(a=0.05)

c ND not detected

3 Discussion

Some soilborne fungi, which are potentially useful as biocntrol agents, are known to secret chitinolytic enzymes and endoglucanases *in situ*[5]. Production of extracellular β-1,3-glucanases, chitinases, and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls[6]. These observations, together with the fact that chitin, β-1,3-glucan and protein are the main structural components of most fungal cell walls, are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens[4].

Fig. 1 *Trichoderma* MM35 parasite *R. solani* (× 400)

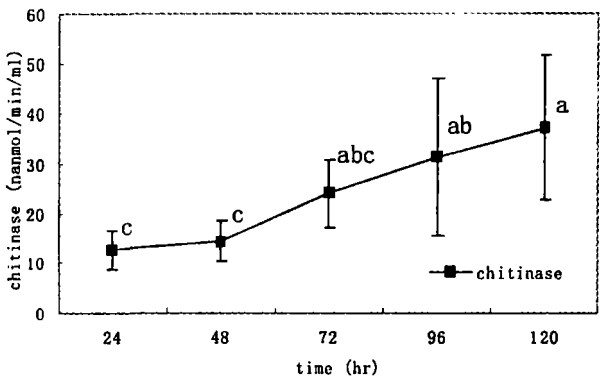

Fig. 2 Activity of chitinase produced by *Trichoderma* MM35 in shaking culture

The levels and order of enzymes production by *Trichoderma* MM35 induced by dried *F. oxysporum* inoculum as carbon source reflected the structure and contents of chitin and β-glucan in the cell wall of *F. oxysporum*. The result showed that the production of β-1,3-glucanases was induced before that of chitinases, whilst the levels of β-1,3-glucanase production was more higher than that of chitinase. Changes in soil enzymes activities showed that pathogen *F. oxysporum* could somewhat repress the production of β-1,3-glucanase (day 9), while production of chitinase was not repressed. All of these suggested that the content of β-glucan was more than that of chitin in the cell wall of *F. oxysporum*, and the chitin layers in cell wall appeared to be buried in β-glucan, rendering little chitin to expose outside.

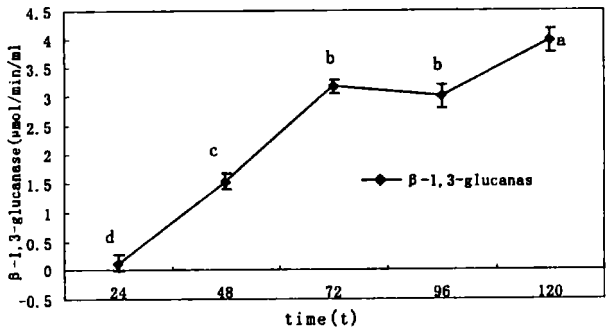

Fig. 3 Activity of β-1,3-glucanase produced by *Trichoderma* MM35 in shaking culture

Enzymes productions were repressed with fresh mycelium of *F. oxysporum* in soil; however, there was a continuous increase in enzymes production with dried mycelium of *F. oxysporum*. The effect might be a result of some metabolites produced by pathogen *F. oxysporum* against antagonists, the mechanism by pathogen *F. oxysporum* against antagonists required further investigation.

Cotes et al. (1994) showed a significant correlation between the ability of several isolates of *Trichoderma* to control *R. solani* in bean and chitinases activities[7]. Although there was no parasitism in interaction of *Trichoderma* MM35 with *F. oxysporum* in vitro, chitinase activity was still significantly higher in soil infested with *F. oxysporum* and *Trichoderma* MM35 *in vivo*, and activities of chitinase and β-1,3-glucanase were significantly higher in soil infested with *R. solani* and *Tri-*

choderma MM35. Extracellular hydrolytic enzymes produced by *Trichoderma* spp. may assist antagonists in warding off phytopathogens. The increases in hydrolytic extracellular enzymes activities in soil caused by inoculation of *Trichoderma* MM35 in presence of pathogens could be related to biological control activities.

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木霉菌(胞外水解酶)拮抗大豆根腐病病原菌的机制研究

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摘要　通过室内试验与温室试验研究了具有生防能力的木霉菌株 *Trichoderma* MM35 所分泌的胞外水解酶在拮抗大豆根腐病病原菌(*F. oxysporum*、*R. solani*)中的作用。试验结果表明:以病原菌 *F. oxysporum* 烘干的菌丝体作唯一碳源, 可以诱导 *Trichoderma* MM35 分泌几丁质酶、β-1, 3-葡聚糖酶。β-1, 3-葡聚糖酶高水平诱导表达在前, 几丁质酶诱导表达在后。土壤中接种 *Trichoderma* MM35、*F. oxysporum* 和 *R. solani* 之后都能够检测到几丁质酶、β-1, 3-葡聚糖酶活性。向有病原菌 *F. oxysporum* 的土壤中接种 *Trichoderma* MM35, 土壤中几丁质酶活性能够显著升高。向有病原菌 *R. solani* 的土壤中接种 *Trichoderma* MM35, 土壤中的几丁质酶、β-1, 3-葡聚糖酶活性都显著升高。

关键词　木霉菌; 大豆根腐病; 机制; 胞外酶; 几丁质酶; β-1, 3-葡聚糖酶