

## Short Communication

# PCR cloning and heterologous expression of chitinase gene of endophytic *Streptomyces aureofaciens* CMUAc130

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Chitin, a  $\beta$ -(1,4)-linked *N*-acetylglucosamine (GlcNAc) polymer, is one of the most abundant and important sources of nutrients and energy in nature. Actinomycetes are well-known decomposers of chitin in soil (Gooday, 1990). Chitinase (EC 3.2.1.14) and  $\beta$ -*N*-acetylglucosaminidase (EC 3.2.1.30) are essential components catalyzing the conversion of insoluble chitin to its monomeric component. These enzymes are found in a wide variety of organisms including bacteria, fungi, insects, plants, and animals. We have investigated the chitinolytic enzyme of the endophyte *Streptomyces aureofaciens* CMUAc130, which can be used for fungal cell wall degradation. The chitinases obtained from *Streptomyces* have been reported for antifungal activity (Tsuji et al., 2000). In this paper, we describe the PCR cloning and sequencing of *Chi40* from *S. aureofaciens* CMUAc130 and the characterization of its translated products. We also show the activity of *Chi40* in the hydrolysis of insoluble chitin and its antagonism against phytopathogenic fungi.

Chromosomal DNA, of endophytic isolate *S. aureofaciens* CMUAc130, was extracted according to the

method of Hopwood et al. (1985). Two oligonucleotide primers, 5'-TTGACCCAGTGGTCCAGACC-3' (forward primer) and 5'-GTGTGCTGCTCAGGCCAG-3' (reverse primer), designed from the conserved regions among the family 19 chitinase genes of *Streptomyces* sp. were used. A standard PCR was performed in a total volume of 100  $\mu$ l, containing 100 mM (each) deoxynucleotide triphosphate (dATP, dCTP, dGTP, dTTP), 50 pmol of each PCR primer, 1.5 U of *Pfu* DNA polymerase (Promega Co., Madison, WI, USA) and 50 ng of genomic DNA from *S. aureofaciens* CMUAc130. A PCR utilized melting 94°C 5 min, then 35 cycles of 94°C 30 s, 55°C for 1 min, and 72°C extension 1 min, with extension at 72°C for 10 min after the last cycle. The purified PCR products were ligated into the *Sma*I site of pUC18 producing the clone, pChi40\_Sau. The nucleotide sequence presented in this article will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB106648.

The complete 1.4-kb sequence of the insert was determined, and the deduced amino acid sequence of Chi40\_Sau yielded an open reading frame of 1,242 nucleotides, coding for a protein of 413 amino acids with an estimated molecular mass of 40 kDa (Fig. 1). A database search revealed that the deduced Chi40\_Sau protein amino acids sequence was 87, 87, 31, 29, and 28% identical to those of Chit\_Strpl from

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	<u>TTGAGC</u> CAGTGGTCCAGACCTTCTA <u>TATTCG</u> CGCCACGGGCGTGCTGACCGTCATGCCCTGACATCCCCCGCACACAGAGGAGCG	90
1	CTTCATGCGCTTCAGACACAAAGCCGCGGCACTCGCAGCGACCTGGCGCTTCCCCTCGCGCGCTGGTGGCCCTCGCGAGCCCGGCCCA	180
	M R F R H K A A A L A A T L A L P L A G L V G L A S P A Q	
	GGCGGCCACACGCGCGACCCCTTCCAGAAGACCTCGGACTGGGCGACGGTCAAGCTGGGCTACTTCACCAACTGGGCGGT	270
30	A A T S A T A T F Q K T S D W G T G T V K L G Y F T N W G V	
	CTACGGGCGCAACTACCAGTGAAGAACCTGGTCACCTCCGGCTCCGCGACAAGATCACGCACATCAACTATGCCTTCGGCAACGTCCA	360
60	Y G R N Y H V K N L V T S G S A D K I T H I N Y A F G N V Q	
	GGCGGGCAAGTGCACCATCGGCGACTCCTACGCGGACTACGACAAGGCGTACACCGCGGACAGTCCGTGACGGCGTCGCGGACACCTG	450
90	G G K C T I G D S Y A D Y D K A Y T A D Q S V D G V A D T W	
	GGACACGCGCTGCGCGCAAACTTCAACCAGTGCAGGTTGAAGGCCAAGTACCGCACATCAAGGTCTCTACTCTTCGGCGGCTG	540
120	D Q P L R A N F N Q L R K L K A K Y P H I K V L Y S F G G W	
	GACCTGGTCCGGCGGCTTCCCGACGCGGTGAAGAACC CGCGCGCTTCGCGCAGTCTGCTACGACCTGGTGGAGGACCCGCGCTGGGC	630
150	T W S G G F P D A V K N P A A F A Q S C Y D L V E D P R W A	
	CGACGTCTTCGACGGCATCGACTGGGAGTACCCCAACGCTCGGCTCTAGCTGTGACACGACGCGCCCAACGCTTCAGCAACATGAT	720
180	D V F D G I D W E Y P N A C G L S C D T S G P N A F S N M M	
	GAAGGCGTGC GCGCGGAGTTCGGCGACAACCTGGTCACCGCGGCGCTCACCTCCGCGACGCGATAGCGAAGTCAAGGCCAAGGCGTCCC	810
210	K A V R A E F G D N L V T A A V T A D G S D G G K I D A T D	
	CTACGGCGAGGCTCGAAGTACATCGACTGGTACAACGTGATGACGTACGACTTCTTCGGCGCTGGGCGAAGAACGGCCGACCGCCCC	900
240	Y G E A S K Y I D W Y N V M T Y D F F G A W A K N G P T A P	
	GCACTCGCGCTCACCTCGTACGACGGCATCCCGCAGCAGGCTTCACCTCCGCGACGCGATAGCGAAGTCAAGGCCAAGGCGTCCC	990
270	H S P L T S Y D G I P Q Q G F T S A D A I A K F K A K G V P	
	GGCGGACAAGCTCCTGATCGGCATCGGCTTCTACGGCGCGGCTGGACGGCGTCACGCGAGTCCGCGCCGGCGGCGACCGCCACCGGCC	1080
300	A D K L L I G F Y G R G W T G V T Q S A P G G T A G P	
	GGCGGCGGCGACCTACGAGGCGCGCATCGAGGACTACAAGTCTCAAGAACAGCTGCCCGGCCACCGGACCGTCCGCGGCGACCGCGTA	1170
330	A A G T Y E A G I E D Y K V L K N S C P A T G T V A G T A Y	
	CGCCCACTGCGGCTCCAAGTGGTGGATCTACGACACCCCGGACACCATCAAGTCAAGATGGACTGGGCGAAGAGAGAGGTCTCGGCGG	1260
360	A H C G S N W W I Y D T P D T I K S K M D W A N E Q G L G G	
	CGCTTCGTGGGACTTCAGCGGCGACACCGCGAAGCGCAACTGGTGAGCGCCATCAACAGCGGCGCTGGCGTGAGCAGCACAC	1347
390	A F V W D F S G D T A N G E L V S A I N S G L A *	

Fig. 1. Nucleotide sequence of the 1.35-kb DNA fragment and the deduced amino acid sequence of Chi40\_Sau.

The putative ribosome-binding site (AGGAGG) is underlined. The -10 and -35 regions of a possible promoter sequence are boxed. The signal peptide cleavage site is shown with an arrow (↓). The stop codon is indicated by an asterisk (\*). The doubly underlined nucleotide sequences denote the conserved regions of *Streptomyces* sp. family 19 chitinase used to design the PCR primers.

*S. plicatus* ATCC27800 (Robbins et al., 1992), Chia\_Strth from *S. thermoviolaceus* OPC-520 (Tsujiibo et al., 1993a), Chi1\_Bacci from *Bacillus circulans* WL-12 (Watanabe et al., 1993), Chia\_Altso from *Al-teromonas* sp. strain O-7 (Tsujiibo et al., 1993b) and Chia\_Serma from *Serratia marcescens* (Jones et al., 1986) (Fig. 2), respectively. These comparisons suggest that the levels of diversity between various chitinases correlate with the evolutionary distances between the bacteria that produce them. The precursor protein of Chi40\_Sau had a leader peptide identical to that of Chit\_Strli (Miyashita and Fujii, 1993) and Chit\_Strpl (Robbins et al., 1992), suggesting that it is also cleaved at the same site. Thus, the mature protein probably starts at Ala31. The type-III homology unit of fibronectin found in *Chi63* and *ChiA* does not exist in the amino acid sequence of Chi40\_Sau. The fibronectin-like sequence is also not found in *Chi40* from *S. thermoviolaceus* OPC-520 (Tsujiibo et al., 1993a) nor the chitinase gene from *S. erythraeus* (Kamei et al., 1989). We also did not find this fibronectin-like sequence in this gene. These results indicated that the fibronectin type-III sequence was not essential for chitinase activity. The amino acid sequence homology could be found between Chi40\_Sau

and *Chi40* from *S. thermoviolaceus* OPC-520 except for a short region which may be involved in the heat stability. The *S. aureofaciens* chitinase gene characterized in this paper has high DNA sequence homology (95%) with *Chi40* encoding the chitinase of *S. thermoviolaceus* OPC-520. Identity between the two chitinases at the amino acid level was 87%. Although the amino acid sequence of Chi40\_Sau is very similar to that of *Chi40* from *S. thermoviolaceus* OPC-520, the heat stability of the two enzymes is clearly different. It was reported that *Chi40* from *S. thermoviolaceus* OPC-520 is stable when heated in 50 mM Tris-HCl (pH 8.0) at 80°C for 30 min (Tsujiibo et al., 1993a). In contrast, Chi40\_Sau showed 25% remaining activity after being heated in the same solution at 55°C for 30 min, but was absolutely inactivated when heated at 65°C for 1 min (data not shown). These results indicate a short region of amino acid sequence which differs from *Chi40* of *S. thermoviolaceus* OPC-520 and may be involved in heat stability. One of the regions (amino acid 156–158 of Chi40\_Sau) contains an Asp and Glu present in the active site of lysozyme (Metraux et al., 1989). These two portions of Chi40\_Sau may therefore constitute the catalytic site of *Chi*, as described in several papers (Kuranda and Robbins, 1991; Tsujiibo

Chia_Strau	-----	
Chit_Strpl	HWTAKNVGNGTLAPGASVSFGFNGSGPGSPSGCKINGGSCDSSV <b>PGDEAP</b> SAPGTPTA	120
Chia_Strth	-----	
Chi1_Bacci	-----	
Chia_Altso	VYFDDLLVNQGS <b>L</b> PAGTKSGVVQFPYTKSGRHQLYLELCEGTVCARSAGKEIIVADTDG-	115
Chia_Serma	ILLNGKEAWSG--PSTGSSGTANFKVNKGGRYQMQVALCNADGCTASDATEIIVVADTDG-	112
Chia_Strau	-----	
Chit_Strpl	<b>SNITDTSVKLSWSAATDDKG</b> VKNYDVLRDGATVATVTGTTT <b>Y</b> T <b>D</b> NGLTKGTDYSY <b>S</b> VKARD	180
Chia_Strth	-----	
Chi1_Bacci	-----	
Chia_Altso	-----	
Chia_Serma	-----	
Chia_Strau	-----VKLG <b>Y</b> FTNWGVYGRNYHVKNLVTSGSADK	47
Chit_Strpl	<b>TGDQTGPASGSVKVTTTGGD</b> GEPNPN <b>GA</b> EVKMGYFTNWGVYGRNYHVKNLVTSGSAEK	240
Chia_Strth	-----VKLG <b>Y</b> FTEWGTYDRNFNVKNLDTSGTAAK	36
Chi1_Bacci	-----DSYK <b>I</b> VGYYPSWAA <b>Y</b> GRNYNVADIDPT----	28
Chia_Altso	-----AHLAPLPMNVDPNNRNGTIPGRVTGAYFVEWGIYGRNYDVT <b>KIPAHN</b> ----	163
Chia_Serma	-----SHLAPLKEPLLEKNKPYQNSGKVVG <b>S</b> YFVEWGVYGRNFTVDK <b>IP</b> AQN----	160
Chia_Strau	ITHINYAFG----NVQGGKCTIGDSYADYDKAYTADQ <b>S</b> VDG-----	84
Chit_Strpl	ITHINLRFG----NVQGGKCTIGDAYADYDKAYTADQ <b>S</b> VDG-----	277
Chia_Strth	ITHINYAFG----NVTGGKCAIGDSYADYDKAYTADQ <b>S</b> VSG-----	73
Chi1_Bacci	VTHINYAFADICWNGIHGNPDPSGPNPVTWTCQNEKSQTINVPGTIVLGD <b>PWIDT</b> KGTF	88
Chia_Altso	LSHILYGFIPICGPN---ESLKSIEIGNSWRALQTACADSQD-YEVV <b>I</b> HDPWAAVQK <b>S</b> MP	219
Chia_Serma	LTHLLYGFIPICGNGINDSLKEIEG--SFQALQRSCQ <b>G</b> RED-FKVS <b>I</b> HDPFAALQK <b>A</b> QK	217
Chia_Strau	VADTWDQPLRANFNQLRLKAKYPHIKVL <b>Y</b> SFGGWTWSGGF <b>P</b> DAVKNPAAFAQSCYDLVE	144
Chit_Strpl	VADTWDQPLRANFNQLRLNKA <b>E</b> YPHIKIL <b>Y</b> SFGGWTWSGGF <b>P</b> DAVKNPAAFAKSCHDLVE	337
Chia_Strth	QADTWDQPLRANFNQLRLKAKYPHIKVL <b>W</b> SFGGWTWSGGFADAAK <b>R</b> PAAFQSCYNLVH	133
Chi1_Bacci	AGDTWDQPIAGNINQLNKLKQTNPNLKT <b>I</b> ISVGGWTWSNRFS <b>D</b> VAA <b>T</b> -AATREV <b>F</b> ANS <b>A</b> V	147
Chia_Altso	GVD <b>A</b> KD-PIRGV <b>Y</b> SQLMALKQRY <b>P</b> DLKILPSVGGWTLS <b>D</b> PFHGF <b>T</b> NK-ANRDT <b>F</b> VAS <b>V</b> KQ	277
Chia_Serma	GVTAWDDPYKGNFGQLMALKQAH <b>P</b> DLKILPSIGGWTLS <b>D</b> PF <b>F</b> FMGDK-VK <b>R</b> DRFVGS <b>V</b> KE	276
Chia_Strau	DPRWADVF <b>D</b> GID--WEY <b>P</b> NACGLSCD--TSGPN-----AFS <b>N</b> MMKAVRAEF <b>G</b> -DNL <b>V</b> TA	193
Chit_Strpl	DPRWADVF <b>D</b> GIDLDWEY <b>P</b> NACGLSCDE-TSAPN-----AFSS <b>M</b> MMKAMRAEF <b>G</b> QDYL <b>I</b> TA	390
Chia_Strth	DPRWDGV <b>F</b> DGID--WEY <b>P</b> NACGLTCD--SSGPD-----AFR <b>N</b> LMAAVRST <b>F</b> G-DEL <b>V</b> TA	182
Chi1_Bacci	DFLRKYN <b>F</b> DGV <b>D</b> LWEY <b>P</b> VSGGLDGNS-KRPEDKQNYTLLSKIREKLDAAGAVDGK <b>K</b> YL	206
Chia_Altso	FLKT <b>W</b> KFYDGV <b>D</b> IDWE <b>F</b> PGGDGPN <b>P</b> DLGDPINDGPAYVALMQELRAMLD <b>E</b> LEA <b>T</b> GRQ <b>Y</b> E	337
Chia_Serma	FLQT <b>W</b> KFFDGV <b>D</b> IDWE <b>F</b> PGGKGAN <b>P</b> NLGS <b>P</b> Q-DGETYVLLMKELRAMLD <b>Q</b> LSA <b>E</b> TGR <b>K</b> YE	335

Fig. 2. Alignment of deduced amino acid sequences of some bacterial chitinases.

The amino acid sequences of the chitinases from *S. aureofaciens* (Chia\_Strau, deduced from translation of the DNA sequence obtained in this study), *S. plicatus* (Chit\_Strpl, from the Swiss-Prot database, accession no. P11220), *S. thermoviolaceus* (Chia\_Strth, from the GenBank DNA sequence, accession no. JC2135), *B. circulans* (Chi1\_Bacci, from the Swiss-Prot database, accession no. P20533), an *Alteromonas* sp. (Chia\_Altso, from the Swiss-Prot database, accession no. P32823) and *S. marcescens* (Chia\_Serma, from the Swiss-Prot database, accession no. P07254) were aligned by using the Clustal W 1.82 program (European Bioinformatics Institute, Cambridge, UK). Numbering starts from the first residue of the mature protein. Asterisks indicate identical residues in all proteins shown. The type-III homology unit of fibronectin is underlined. The amino acid Asp156 and Glu158 that are identical in the active site of lysozyme are in boldface.

et al., 1993a; Watanabe et al., 1992).

Most of the cloned chitinase enzymes have been found to accumulate in the periplasmic space of the host cells (Robbins et al., 1988; Tsujibo et al., 1993b). In contrast *S. aureofaciens* CMUAc130 chitinase is mostly detected in the culture medium when it was cloned in *E. coli*. This experiment indicated that the signal peptide could be functional in *E. coli*, which has been noted in previous reports by Tsujibo et al. (2000) and Li et al. (2000). The chitinolytic activity found in the culture medium of the transformant JM109/pChi40\_Sau was active in the hydrolysis of the colloidal chitin with 1 mM IPTG but very low in the ab-

sence of IPTG (Table 1). These data indicate that the chitinase was regulated by the *lacZ* promoter.

To demonstrate directly that the transformed *E. coli* strain produced and secreted the Chi40\_Sau chitinase, the immunological detection of secreted proteins of JM109/pChi40\_Sau renaturated following separation by SDS-PAGE was examined and compared with that of the original *S. aureofaciens* CMUAc130. The results obtained by Western-blot analysis showed that the specific protein, with an apparent molecular mass of 40 kDa, which is present in the secreted proteins of strain CMUAc130 (Fig. 3, lane 1), corresponding to the 40 kDa chitinase, was detected in the secreted pro-

Table 1. Chitinase activity of *E. coli* JM109 carrying pChi40\_Sau in LB after overnight growth at 37°C and then adding 1 mM IPTG 3 h before harvesting.

Source of culture medium	Chitinase activity of heterologous expression (U ml <sup>-1</sup> ) <sup>a</sup>	
	1 mM IPTG	No IPTG
<i>E. coli</i> JM109/pChi40_Sau		
Secreted proteins <sup>b</sup>	0.274±0.058	0.079±0.023
Intracellular proteins <sup>c</sup>	0.106±0.072	0.014±0.021
<i>E. coli</i> JM109/pUC18 <sup>b</sup>	0.000	0.000

<sup>a</sup> The cultured broth and the sonicated cells were subjected to quantitate chitinase activity. This was quantitated by the method described by Reissig et al. (1955). Chitinase activity was calculated and expressed as unit per ml of the original volume of cell cultures.

<sup>b</sup> The secreted proteins were the total soluble proteins from the cultured broth.

<sup>c</sup> *E. coli* JM109 carrying pChi40\_Sau was grown in 100 ml LB broth containing ampicillin (100 µg ml<sup>-1</sup>) at 37°C overnight. Cell culture was harvested by centrifugation at 8,220×*g* for 10 min at 4°C. The pellets were resuspended in 5 ml of sterile distilled water and sonicated.

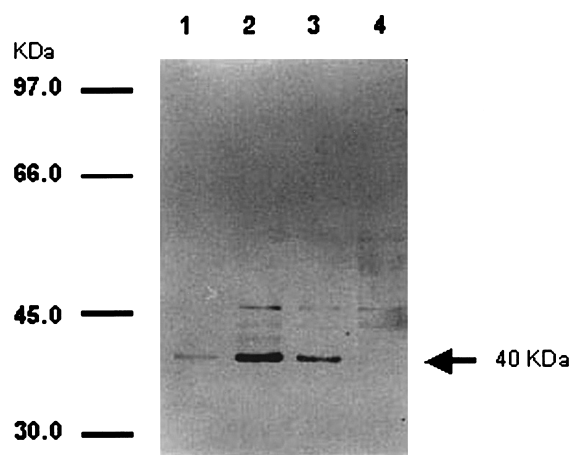


Fig. 3. Western-blot analysis showing the expression of the Chia\_Strau gene.

Fifty micrograms of total soluble protein was loaded per lane. Lane: 1, crude chitinase from *S. aureofaciens* CMUAc130; 2, secreted protein from *E. coli* JM109/pChi40\_Sau with 1 mM IPTG induction; 3, secreted protein from *E. coli* JM109/pChi40\_Sau without IPTG induction; 4, secreted protein from *E. coli* JM109.

teins of JM109/pChi40\_Sau with IPTG induction (Fig. 3, lane 2). No bands of immunological reactivity from the secreted proteins of *E. coli* JM109 were found (Fig.

3, lane 4).

Antifungal activity was estimated using the hyphal extension-inhibition assay of Roberts and Selitrennikoff (1986). *Fusarium oxysporum*, the causative agent of wilt of wheat was used as the test fungus. The chitinolytic *E. coli* strain carrying the plasmid pChi40\_Sau was found to suppress the growth. The original strain CMUAc130 showed stronger suppression of the fungi, whereas almost no suppression was observed with the nontransformed *E. coli* strain.

The effects of crude preparations of the chitinases secreted by the original *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40\_Sau on spore germination of *F. oxysporum* were studied according to the procedure described by Lorito et al. (1993), using *F. oxysporum* as the test fungus and the crude secreted enzyme preparation from *S. aureofaciens* CMUAc130, *E. coli* JM109/pChi40\_Sau, or *E. coli* JM109 as the test solutions. A stock conidial suspension, 10<sup>5</sup> spores ml<sup>-1</sup> in 30% glycerol, was prepared and kept at -20°C. Equal volumes (20 ml) of spore suspension, 3× potato dextrose broth, and the test solution (about 5 U of chitinase activity) were mixed in a sterile flask. The tested solutions were replaced with sterile water in control samples. Flasks were incubated at 30°C for 24 h. A drop of the mixture from each flask was placed on a microscope slide, and the percentage of conidial germination was determined from the first 100 spores chosen at random.

The rate of spore germination in the control was 80.6%±8.0%. The *F. oxysporum* spore germination assay showed that in the presence of crude preparations of secreted proteins produced by strain CMUAc130 and JM109/pChi40\_Sau, the rate of spore germination significantly decreased relative to the control (39.0%±7.5% and 56.6%±5.5%, respectively). When the amount of secreted proteins of the parent strain JM109 equivalent to that in crude preparations of chitinases secreted by strain CMUAc130 and JM109/pChi40\_Sau was added to the experimental mixture as a test solution, almost no difference from the control was found (79.0%±4.5%). Morphological changes were observed in microslide-culture of *F. oxysporum* treated with crude chitinase of *E. coli* JM109/pChi40\_Sau (5 U ml<sup>-1</sup>) at 37°C for 12 h in a moist chamber compared with a 5 min-boiled chitinase control under the light microscope (400×). This treatment caused fungal cell wall lysis, as well as inhibition of spore germination and, possibly, conidial damage.

This may be due to enzymatic digestion of the cell wall region rich in chitin. Hyphal width reduction was also observed. Different morphological effects on the fungal cell wall have been reported in studies using *Streptomyces* sp. or other microbial chitinases. These effects were mainly spore germination inhibition, bursting of spore and hyphal tips and germ tube elongation (Gomes et al., 2001). The differences in the effects can be attributed to the different experimental conditions of the tests.

*E. coli* JM109/pChi40\_Sau, expressing the *S. aureofaciens* *Chi40* gene, acquired the ability to suppress growth of *F. oxysporum* and their spore germination in vitro. The transformant effected less significant fungal suppression in vitro than did the parent strain CMUAc130. This difference can be explained not only by the lower level of chitinolytic activity secreted by the transformant (probably because the *chiA* gene was cloned under the control of the relatively weak *lac* promoter) but also by the fact that the parent strain has been found to produce other chitinolytic enzymes and other antifungal substances.

The results presented in this work confirm the role of chitinases in the antifungal activity of various microbial antagonists (Lorito et al., 1993; Ordentlich et al., 1988; Shapira et al., 1989; Tsujibo et al., 2000). The *S. aureofaciens* CMUAc130 chitinase gene could therefore be used to transform other bacteria or plants and provide them with the ability to control fungal phytopathogens. The application of *S. aureofaciens* CMUAc130 and *E. coli* JM109/pChi40\_Sau to soil or plants infested with *F. oxysporum* will be reported elsewhere.

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