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e-Xtra*

Symptom Development in Response to Combined Infection of In Vitro-grown *Lilium longiflorum* with *Pratylenchus penetrans* and Soilborne Fungi Collected from Diseased Roots of Field-grown Lilies

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Abstract

Eight fungal isolates (ELRF 1 to 8) were recovered from necrotic roots of Easter lilies, *Lilium longiflorum* cv. Nellie White, grown in a field in the U.S. Pacific Northwest. The eight fungal isolates were identified by sequencing and molecular phylogenetic analyses based on their ITS rDNA region. Five isolates were identified as *Fusarium oxysporum*, two as *F. tricinctum*, and one as *Rhizoctonia* sp. AG-I. This constitutes the first report of *Rhizoctonia* sp. AG-I infecting lilies worldwide and the first report of *F. tricinctum* infecting lilies in the United States. To study and validate their pathogenicity, pure cultures of each isolate were used to infect the roots of Easter lily plants growing in vitro. In addition, Easter lily plants growing in vitro were infected either with or without *Pratylenchus penetrans*, the root lesion nematode, prior to placing a culture plug of fungus 1 cm from a lily root. *Pratylenchus penetrans* is a nematode species commonly found in the sampled fields. The presence of both nematode and *Rhizoctonia* sp. AG-I isolate ELRF 3 in infected lilies was evaluated by molecular analyses, confirming the infection of roots 3 days after inoculation, prior to development of disease symptoms. Necrosis and root rot developed more rapidly with all eight fungal isolates when there had been prior infection with *P. penetrans*, the major nematode parasitizing Easter lily roots in the field in Oregon.

Lilies are an important flower crop grown in the garden and sold as a cutflower. *Lilium longiflorum* cv. Nellie White, known as the Easter lily, is widely sold as a pot plant in the United States with a wholesale value of $24,317,000 (USDA-NASS). In Israel, Easter lilies are produced in the greenhouse and sold mainly as cut flowers, and assume particular economic importance as they represent 90% of the lilies grown in that country (Lebiush-Mordechai et al. 2014). The major fungal pathogens known to infect *Lilium* spp. and cause root rot in The Netherlands, United States, China, and Israel are *Fusarium oxysporum*, *Pythium oligandrum*, and *Rhizoctonia solani* (Baayen et al. 1998; Bald et al. 1983; Conijn 2014; Lawson 2011; Lebiush-Mordechai et al. 2014; Li et al. 2013; Rajmohan et al. 2011; Shang et al. 2014). In addition, both *Rhizoctonia* sp. AG-A and *Fusarium proliferatum* have been reported to cause severe disease in Easter lilies grown in Israel (Lebiush-Mordechai et al. 2014).

Pratylenchus penetrans, the root lesion nematode, is capable of infecting at least 400 plant species (Castillo and Vovlas 2007), including lilies. It is considered a limiting agent for Easter lily production in The Netherlands, Korea, and the United States (Conijn 2014; Kang et al. 2013; Westerdahl et al. 1998). Symptoms of infection on lilies caused by *P. penetrans* include decreased growth of shoots, chlorotic shoots, and sometimes shoots that do not emerge from the bulbs. Infected roots exhibit retarded growth with typical root lesions distributed along the entire root system (Overman 1961). *Pratylenchus* spp. feed by entering and migrating intracellularly throughout root cortical cells and then exiting the root (Castillo and Vovlas 2007). This destructive behavior causes extensive physical damage to cortical and epidermal cells along the length of a root resulting in necrosis, lesions, and death of root cells.

Combined infection with root lesion nematodes and fungus often results in synergistic interactions, wherein the crop loss is greater than the additive effect of the two pathogens. Interactions specifically involving *P. penetrans* and fungal pathogens have been previously reported, and include potato early dying disease which results from the synergistic interaction of *P. penetrans* and *Verticillium dahliae* (Rotenberg et al. 2004). Infection by each pathogen alone causes mild symptoms in potatoes, but infection by the *P. penetrans*-*V. dahliae* complex results in severe symptoms. Roots of potato become colonized by *V. dahliae* to a significantly greater extent when *P. penetrans* has also infected the roots (Bowers et al. 1996). The interaction between *R. fragariae* and *P. penetrans* is reported to have an additive effect on root rot of strawberries (LaMondia 1999, 2003; LaMondia and Cowles 2005).

Only a limited number of papers have been published concerning plant disease complexes that involve microorganisms from different phyla and commensal-pathogen interactions such as nematode-microbe or insect-microbe (Lamichhane and Venturi 2015). This study identified a complex set of soilborne fungi found infecting *L. longiflorum* growing in a field in the U.S. Pacific Northwest. The symptoms from single fungal isolates, as well as the disease severity from representative isolates in combination with *P. penetrans*, a nematode species that is commonly found in soil of the Pacific Northwest, were investigated in *L. longiflorum* cv. Nellie White to evaluate their effective damage in this floral crop.

Materials and Methods

Isolation of fungi from lily bulb. *Lilium longiflorum* cv. Nellie White bulbs were harvested from the field at the Easter Lily Foundation (Brookings, Curry Co., OR) and sent to the USDA in Beltsville, MD, in March 2015. Sections of necrotic roots were surface-sterilized in 2% sodium hypochlorite for 2 min followed by 70% ethanol for 30 s and then rinsed using sterile water three times. Root sections were cultured on water agar in Petri plates. Hyphal tips of mycelia that emerged from the roots were cultured on potato dextrose agar medium (PDA) (Difco Laboratories, Detroit, MI) and subcultured to establish pure fungal cultures (Mullen 2008).

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ITS rDNA sequencing of fungal isolates. Total genomic DNA was extracted from eight fungal cultures using the DNeasy Plant Mini Kit (Qiagen Inc. Valencia, CA), and the internal transcribed spacer (ITS) sequences were amplified using ITS1 forward primer 5′-TCCGTAGGTGAACCTGCGG-3′ and ITS4 reverse primer 5′-TCCTCCGCTTATTGATATGC-3′ (White et al. 1990) following the protocol described by Hsiang and Dean (2001). PCR products were purified with a QIAquick PCR purification kit (Qiagen), cloned in a TOPO TA cloning vector pCR4-TOPO vector using the TOPO TA cloning kit (Thermo Fisher Scientific, Waltham, MA), and sequenced with vector-specific T3 and T7 primers. After trimming the vector sequences the resulting ITS sequences were compared with accessions in the GenBank nonredundant nucleotide database (NCBI, Bethesda, MD) for related fungal sequences using BLASTn analyses (Altschul et al. 1990). The top BLAST hits identified matches, and additional representative sequences of the identified and closely related fungi were obtained from NCBI. ITS sequences were then analyzed and aligned using the program ClustalW implemented in CLC Main Workbench v.7. Phylogenetic trees were generated by neighbor-joining analyses (Saitou and Nei 1987), and bootstrapping analysis was performed with 1000 replicates.

Infectivity testing of *Rhizoctonia* sp. AG-I ELRF 3 isolate on strawberry and sugarbeet. Sugarbeet 'Detroit Dark Red' seeds were soaked in water overnight and then surface sterilized with 2% sodium hypochlorite for 2 min followed by 70% ethanol for 30 s, rinsed with sterile water three times, surface blotted, and then sown in an autoclaved celite:vermiculite (1:1) mixture in 15-cm-diameter pots in the greenhouse. Seedlings of approximately 4 cm long were washed with running tap water, rinsed 5 times in sterile water, then placed on 4 layers of sterile paper towels soaked in sterile water that were placed in trays at 25°C. Diploid strawberry 'Hawaii 4 F7-3' runners were rooted and grown in the greenhouse under ambient conditions in a mixture of ProMix (Premier Horticulture Inc., Quakertown, PA), Farfard 3B (Conrad Fafard Inc. Agawam, MA), and coarse vermiculite (The Schundler Co., Edison, NJ) (2:1:1) supplemented as needed with dolomitic lime. Plants were watered daily with a dilute solution of Miracle Gro Tomato Plant Food according to the manufacturer's specifications. Rooted runners were rinsed in sterile water 5 times and then placed on 4 layers of sterile paper towels soaked in sterile water in trays at 25°C. Five-millimeter-diameter plugs from the margin of 5-day-old cultures of the *Rhizoctonia* sp. AG-I ELRF 3 isolate were placed near the roots of two sugarbeet and two strawberry seedlings. Development of root rot and necrosis, rotting of the crown, and leaf chlorosis were observed over 15 days.

Interaction of fungal isolates and *Pratylenchus penetrans.* *Lilium longiflorum* cv. Nellie White plants were maintained in vitro on Murashige and Skoog's medium (MS) (Murashige and Skoog 1962) supplemented with 3% sucrose and the following in mg/liter: 1.0 glycine; 100 m inositol; 1.0 thiamine; 0.5 pyridoxine; 0.5 nicotinic acid; and solidified with 0.2% (w/v) Phytagel (Sigma-Aldrich, St. Louis, MO). An isolate of *P. penetrans* (NL 10p RH) initially collected in Beltsville, MD, and provided by the Nematology Laboratory (USDA, Beltsville) was maintained and multiplied in vitro in roots of corn (*Zea mays* L. cv. Iochief) growing in MS agar medium at 25°C in the dark. Infested corn roots were chopped up and, along with the MS agar medium, were placed on a metal sieve with 75-μm openings in a sterile glass dish to collect the nematodes. *Pratylenchus penetrans* traveled from the roots and medium through the sieve and then into the water below the sieve with the aid of gravity. Sterile water supplemented with 50 mg/liter carbenicillin and 50 mg/liter kanamycin was used to fill the dish until it touched the roots and MS agar medium that were supported on the sieve. Five days later, the water containing *P. penetrans* was placed in a 50-ml conical Falcon test tube, centrifuged at 4,000 × g for 4 min, and nematodes at the bottom of the tube were collected.

Easter lily plants were placed on sterilized water-agar medium (1% Difco agar, Difco Laboratories) in Petri plates (100 mm × 25 mm). Roots were inoculated with *P. penetrans* using an average number of 500 nematodes/root. Three days after nematode inoculation (DAI), a 5-mm-diameter plug of fungus was placed 1 cm from the root, and fungus-inoculated lilies were then placed in the dark. Roots were observed and scored by two people as either 0 (no visual sign of infection), 1 (infection beginning, characterized by a small region of light-brown necrosis appearing on root), 2 (definite infection of root, as seen by loss of the root's turgidity and/or brown necrosis present), 3 (advanced infection of root that has dark brown necrosis and/or is rotten in at least 25% of its length), 4 (root is rotten and/or necrotic throughout its length). Each replicate consisted of two lily plants, and 3 to 4 independent biological repetitions were done for each fungal isolate. Statistical significance at $P < 0.05$ was determined using a Student's *t* test to compare the roots inoculated either with or without *P. penetrans* and no fungus, and a second comparison made between roots inoculated with or without *P. penetrans* and inoculated with fungus.

PCR verification of lily root infection by *Rhizoctonia* sp. AG-I isolate ELRF 3 and *P. penetrans.* In vitro-grown plants of *L. longiflorum* cv. Nellie White plants (two plants per Petri plate) were placed on water-agar medium in Petri plates, and 500 *P. penetrans* were placed on the sterile medium next to the root-tip region. Plants were grown in the dark at 25°C for three days prior to inoculation with a 5-mm-diameter plug of *Rhizoctonia* sp. AG-I isolate ELRF 3 taken from an actively growing fungus culture and placed a distance of 1 cm from the middle region of the root. Control plates containing lily plants without *P. penetrans* were inoculated with *Rhizoctonia* sp. AG-I isolate ELRF 3 only. Infected roots were collected 3, 8, and 14 DAI for DNA or RNA isolation.

Genomic DNA was isolated from lily roots by first macerating roots in a Lysing Matrix tube containing one ceramic bead and homogenizing for 15 s using the FastPrep system (QBiogene, Carlsbad, CA) followed by a Fast DNA kit (MP Biomedicals, LLC, Santa Ana, CA) according to the manufacturer's instructions. To detect the presence of the fungus in lily-infected roots, a pair of primers was designed to amplify a 100-bp product: forward 5′-CAGGTGTGTGTGGATGAA TAGA-3′ and reverse 5′-ACCTGCGGAAGGATCATTATT-3′ within the ITS rDNA region. Amplification with Platinum Taq (Thermo Fisher Scientific, Waltham, MA) was performed using a PTC-200 Peltier Thermal Cycler, and the reaction consisted of a denaturation step at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 52°C for 30 s, 72°C for 30 s, and a final extension step of 5 min at 72°C.

Lily roots infected with *P. penetrans* were pulverized for RNA isolation using a Retsch MM400 Mixer Mill (Fisher Scientific) followed by RNA isolation with Qiagen's RNeasy Plant Mini kit (Qiagen). The RNA was treated with RNase-Free DNase (Qiagen) before reverse transcription. Reverse transcription of RNA was performed using the iScript first-strand synthesis kit (Bio-Rad, Hercules, CA) to obtain cDNA, and 100 ng of cDNA were then amplified. Detection of *P. penetrans* RNA from roots of lily infected with nematodes was verified by RT-PCR using a 148-bp region of the *18S* rDNA region of *P. penetrans* with forward primer 5′-ATGAGAGGG CAAGTCTGGTG-3′ and reverse primer 5′-GAAAGCCGAAGA CATCCAGT-3′. PCR amplification of the lily *P-450-like TBP* gene encoding a cytochrome P450 protein with forward primer 5′-CCCACTTATCCTACACCTCTCAA-3′ and reverse primer 5′-CCCACTGTCCCTGTCTACTATCC-3′ (Luo et al. 2015) was used for plant gene detection. The conditions for amplification consisted of a denaturation step at 94°C for 2 min followed by 30 cycles at 94°C for 30 s, 57°C for 30 s, 72°C for 30 s, and a final extension step of 5 min at 72°C. PCR products were separated through a 1.5% agarose gel containing SYBR Green 1 Nucleic Acid Stain (Thermo Fisher Scientific) for visualization.

Results

Identification of fungal isolates associated with lily roots. A total of 8 fungal colonies, randomly numerated as ELRF 1 to ELRF 8, were established from hyphal tips of mycelia that emerged from diseased lily roots collected from a lily field from Oregon (Suppl. Fig. S1). The isolation and establishment of the different fungal cultures were initially based on morphological observations. Fungal isolate ELRF 3 was a light to slightly brown color on PDA medium. Under the microscope, its hyphae were observed to be branched at right

angles and the septum was present near the point of branching. Two nuclei were observed in each cell upon staining with lactophenol blue; thus ELRF3 was tentatively identified as a binucleate *Rhizoctonia* (syn. *Ceratobasidium* spp.) (Sneh et al. 1998).

Isolates ELRF 1, ELRF 2, ELRF 5, ELRF 7, and ELRF 8 were identified as *F. oxysporum* and ranged from light to dark violet with older cultures producing a dark-violet pigment in the PDA medium. Isolates ELRF 5 and ELRF 8 produced profuse macroconidia, microconidia, and single chlamydospores. The macroconidia were falcate and five-celled, and the apical cells were tapered and slightly curved. Basal cells were notched to foot-shaped. Microconidia were ovoid and single-celled, and chlamydospores were thick-walled and produced singly. No conidia were observed for isolates ELRF 1, ELRF 2, and ELRF 7, and only a few thick-walled and solitary chlamydospores were found. The lack of spore formation in ELRF 1, ELRF 2, and ELRF 7 is not uncommon because many isolates of *Fusarium* species require specialized media such as carnation leaf (Fisher et al. 1982), wheat bran, or oatmeal agar (Hassan and Bullerman 2009) for macroconidia formation.

ELRF 4 and ELRF 6 grew as white, aerial, dense mycelia on PDA. The 8- to 10-day-old mycelium turned pink, and subsequently the medium turned pink. On PDA plates, ELRF 4 and ELRF 6 formed falcate, 3- to 5-septate hyaline macroconidia, which were curved to lunate with a foot-shaped basal cell and a tapering apical cell. Globose chlamydospores with a smooth cell wall formed in chains. These morphological features of ELRF 4 and ELRF 6 identified the two isolates as *F. tricinctum* (Leslie and Summerell 2006).

The ITS rDNA region, containing both ITS1 and ITS2 domains and the 5.8S rDNA gene, is a commonly used locus for species and fungal strain identification (Capote et al. 2012; Iwen et al. 2002) and was amplified from each individual fungal culture using PCR. The identity of each of the eight fungal colonies collected was determined by sequence similarity using BLASTn analysis at NCBI NR-nucleotide database, and results are presented in Table 1. Sequence data supports the identification of ELRF 3 as binucleate (teleomorph: *Ceratobasidium* sp.) *Rhizoctonia* sp. AG-I. Neighbor-joining phylogenetic analysis based on the ITS sequences obtained for *Rhizoctonia* spp. is shown in Figure 1, clustering isolate ELRF 3 among other worldwide isolates of the established *Rhizoctonia* sp. AG-I group (Fang et al. 2013).

Morphological identification of fungal colonies ELRF 1, ELRF 2, ELRF 5, ELRF 7, and ELRF 8 as *F. oxysporum* was supported by sequencing data, with 99 to 100% similarity within the different isolates. Isolates ELRF 4 and ELRF 6 were identified as *F. tricinctum* (Table 1). Neighbor-joining phylogenetic analysis with the ITS sequences for the *Fusarium* spp. isolates collected from lily roots showed these sequences clustering with either *F. oxysporum* or *F. tricinctum* (Fig. 2), respectively.

Interaction of *Fusarium* spp. isolates with *P. penetrans*. The pathogenicity of different *Fusarium* isolates singly or in combination

Table 1. Top BLAST search results for lily root fungal isolates through the NCBI NR-nucleotide database

Lily fungus isolate no.	ITS sequence length (bp)	Top BLAST hit NCBI sequence (accession no.)	Nucleotide identities (%)	NCBI GenBank accession no.
ELRF 1	546	*Fusarium oxysporum* strain TW9 (KT803066)	99	KX786240
ELRF 2	546	*Fusarium oxysporum* strain TW9 (KT803066)	100	KX786241
ELRF 3	656	Uncultured fungus clone YJ44 (KU931538)	100	KX786242
		Rhizoctonia sp. AG-I isolate WUF-ST-RhT3-12 (JQ859888)	99	
ELRF 4	561	*Fusarium tricinctum* isolate T6 (KT779291)	99	KX786243
ELRF 5	546	*Fusarium oxysporum* strain TW9 (KT803066)	100	KX786244
ELRF 6	561	*Fusarium tricinctum* isolate T6 (KT779291)	99	KX786245
ELRF 7	545	*Fusarium oxysporum* strain DJ1 (KC201696)	100	KX786246
ELRF 8	546	*Fusarium oxysporum* strain TW9 (KT803066)	100	KX786247

Fig. 1. Phylogenetic relationship of *Rhizoctonia* sp. AG-I isolate ELRF 3 collected from infected lilies to other worldwide isolates and groups of *Rhizoctonia*, based on sequence alignment of the ITS region of the rDNA. The phylogram was generated by neighbor-joining analysis with 1000 bootstrap replications. Bootstrap values are indicated at the nodes. Scale bar = 0.05 substitutions/site.

with nematode infection were evaluated using *F. oxysporum* isolates ELRF 1 and ELRF 7 and *F. tricinctum* isolates ELRF 4 and ELRF 6 (Table 2 and Fig. 3A). All *Fusarium* isolates tested were able to establish and cause mild necrotic symptoms scored as 0.41-1.75 at 3 to 8 DAI when inoculated individually in lilies (Table 2). Lily roots inoculated only with *P. penetrans* showed nematode-induced lesions that were scored as 0.17 to 0.75 during the eight days when symptoms were first observed to be different for roots inoculated with fungus and either with or without *P. penetrans* (Table 2). In comparison, roots that had been inoculated with *P. penetrans* followed by inoculation with any of the *Fusarium* isolates exhibited more advanced necrosis and rotting, scored as 2.00 to 2.75 (Table 2). The most dramatic symptoms, necrosis and rotting, were observed after combined infection with *P. penetrans* and *F. tricinctum* isolate ELRF 6. Three of the five *F. oxysporum* isolates (ELRF 1, ELRF 5, ELRF 7) showed a statistically significant higher score for necrosis and rotting following a combined inoculation as compared with a single inoculation with *F. oxysporum* only (Table 2, data not shown for ELRF 2, ELRF 5, ELRF 8). Roots inoculated with all five *Fusarium* isolates in combination with *P. penetrans* consistently had higher mean scores than roots solely inoculated with *Fusarium*, although the difference in scores with and without *P. penetrans* was not always statistically significant.

Interaction of *Rhizoctonia* sp. AG-I isolate ELRF 3 with *P. penetrans* infection. Although *Rhizoctonia* species have previously been reported as capable of infecting lilies, the occurrence of *Rhizoctonia* sp. AG-I has been mainly associated with strawberry and sugarbeet (Yang and Li 2012). Therefore, we conducted a pathogenicity assay using *Rhizoctonia* sp. AG-I isolate ELRF 3 in roots of strawberry and sugarbeet plants (Suppl. Fig. S2). Dark, necrotic lesions appeared on sugarbeet roots inoculated with this isolate by 4 to 5

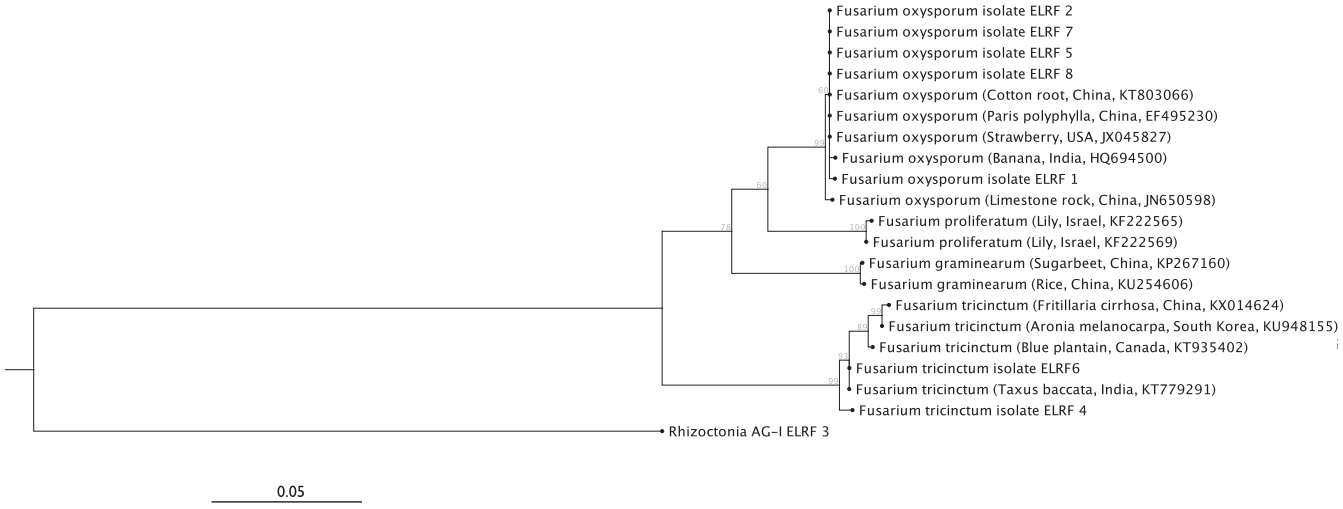

Fig. 2. Phylogenetic relationship of *F. oxysporum* isolates ELRF 1, ELRF 2, ELRF 5, ELRF 7, ELRF 8, and *F. tricinctum* isolates ELRF 4 and ELRF 6 collected from infected lilies, to other worldwide isolates and other species of *Fusarium*, based on sequence alignment of the ITS region of the rDNA. The phylogram was generated by neighbor-joining analysis with 1000 bootstrap replications. Bootstrap values are indicated at the nodes. Scale bar = 0.05 substitutions per site.

Table 2. Visual scores for *L. longiflorum* roots infected with five fungal isolates, either with or without prior infection with the nematode *P. penetrans*

Fungal isolate number	*P. penetrans* (+ or −)	Visual score ± SE	DAI of first symptom difference[z]
None	−	0 a	3 to 5
None	+	0.75 ± 0.31 a	
ELRF 1 (*F. oxysporum*)	−	1.00 ± 0.33 a	
ELRF 1 (*F. oxysporum*)	+	2.12 ± 0.12 b	
None	−	0 a	3 to 5
None	+	0.33 ± 0.21 a	
ELRF 7 (*F. oxysporum*)	−	0.41 ± 0.17 a	
ELRF 7 (*F. oxysporum*)	+	2.33 ± 0.21 b	
None	−	0 a	4 to 6
None	+	0.30 ± 0.15 a	
ELRF 3 (*Rhizoctonia* AG-I)	−	1.11 ± 0.26 a	
ELRF 3 (*Rhizoctonia* AG-I)	+	2.00 ± 0.33 a	
None	−	0 a	3 to 5
None	+	0.17 ± 0.17 a	
ELRF 4 (*F. tricinctum*)	−	0.83 ± 0.31 a	
ELRF 4 (*F. tricinctum*)	+	2.00 ± 0.52 a	
None	−	0 a	4 to 8
None	+	0.50 ± 0.29 a	
ELRF 6 (*F. tricinctum*)	−	1.75 ± 1.03 a	
ELRF 6 (*F. tricinctum*)	+	2.75 ± 0.75 a	

[z] The first DAI when a visible difference in symptoms was observed between roots inoculated with fungus and with or without *P. penetrans*. A range of DAI is shown because this first DAI differed with each replicate. Lily plants were placed on water-agar plates and inoculated with an average 500 *P. penetrans*/plant root at the root meristem region. Three days later a plug of fungus was placed 1 cm from the root. Plants were observed every other day for 14 DAI and scored as described in Materials and Methods. Each trial consisted of two lily plants, and 3-4 independent replicates were done. Means of the visual scores ± standard error are shown. Significance was determined using a Student's *t* test comparing with or without *P. penetrans* (no fungus inoculation) and with or without *P. penetrans* (inoculated with fungus). Scores with a different letter were significantly different ($P < 0.05$) for each isolate.

DAI, and these roots subsequently rotted. In the case of strawberry roots, light to dark brown necrosis, stunting, and rot occurred at 6 to 8 DAI, confirming the infectivity of this isolate to strawberry plants. Also, foliage of inoculated strawberry plants showed mild chlorosis. Control inoculation, using only an agar plug, did not produce any symptoms on roots of either sugarbeet or strawberry.

Infections of lilies with *Rhizoctonia* sp. AG-I isolate ELRF 3 alone, or in combination with *P. penetrans,* were performed as described above for *Fusarium* spp. When inoculated with *Rhizoctonia* sp. AG-I isolate ELRF 3 alone, root necrosis visually scored as 2 at 6 to 8 DAI, confirming its pathogenicity to *L. longiflorum* cv. Nellie White. Dual infection with both *Rhizoctonia* sp. AG-I isolate ELRF 3 and *P. penetrans* resulted in earlier observable symptoms at 4 to 6 DAI (Table 2).

The presence of *Rhizoctonia* sp. AG-I isolate ELRF 3 or *P. penetrans* was validated in infected roots of lilies inoculated with or without nematodes using PCR analysis at 3, 8, and 14 DAI. PCR of fungal genomic DNA and RT-PCR using cDNA from lily-infected roots confirmed the presence of *Rhizoctonia* sp. AG-I isolate ELRF 3 and *P. penetrans*, respectively (Fig. 4). Without prior nematode inoculation, the presence of *Rhizoctonia* isolate ELRF 3 was detected in lily roots at 3 DAI although the roots were still asymptomatic. Levels of fungus appeared to be lower in roots inoculated only with *Rhizoctonia* sp. AG-I at 3, 8, and 14 DAI as compared with roots inoculated with both nematode and fungus, based on the intensity of the PCR product visualized on the gel. In the case of double infection (nematode plus fungus), both *Rhizoctonia* sp. AG-I isolate ELRF 3 and *P. penetrans* were detected in doubly infected lily roots (Fig. 4), and

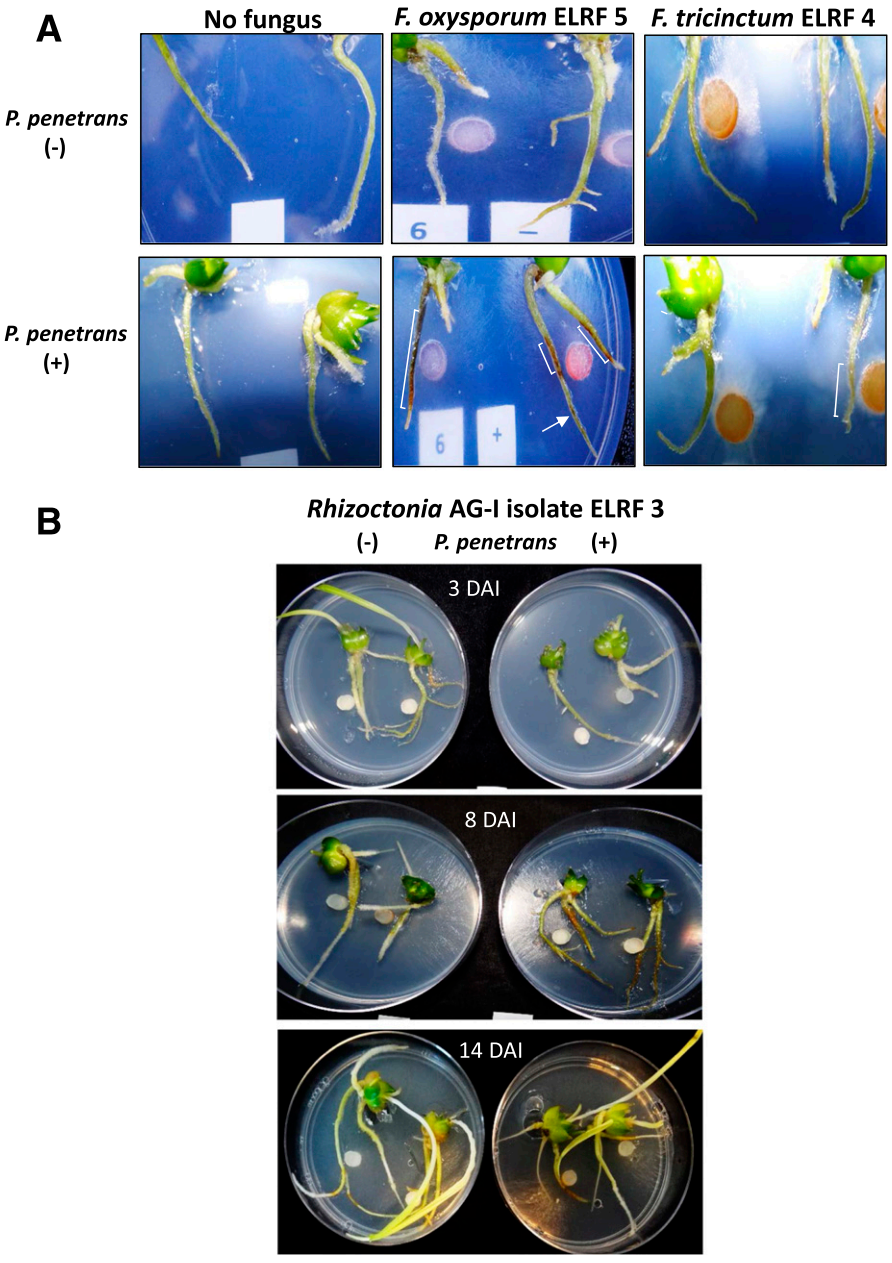

Fig. 3. A, *L. longiflorum* plants inoculated with only *P. penetrans* did not show necrosis at 5 DAI. At 5 DAI necrosis was present in root tips of plants inoculated with both *F. oxysporum* and *P. penetrans* (brackets and arrows), and roots inoculated only with *F. oxysporum* were asymptomatic. Roots inoculated with *P. penetrans* and *F. tricinctum* showed rotting (bracket), while roots inoculated with *F. tricinctum* but not *P. penetrans* were either white or the third root had a light-brown region that was not from lesions or necrosis 5 DAI. **B,** *L. longiflorum* plants analyzed by PCR analysis of root DNA 3, 8, and 14 DAI with *Rhizoctonia* sp. AG-I isolate ELRF 3. Root tips were either inoculated (+) or not inoculated (−) with *P. penetrans* three days prior to inoculation of roots with a 5-mm-diameter plug of *Rhizoctonia* AG-I isolate ELRF 3.

roots showed increased necrotic symptoms at 8 and 14 DAI when compared with roots inoculated with *Rhizoctonia* only (Fig. 3B).

Discussion

Root diseases caused by soilborne pathogens are commonly triggered by a complex of fungi, bacteria, and nematodes, in random or specific associations, infecting simultaneously or in succession, and showing cumulative or synergistic effects (Borer et al. 2016; Lamichhane and Venturi 2015). In this context, lily root rot and lily basal decay (bulb rot) were reported to be associated with single or multiple fungal pathogens in California, The Netherlands, China, and Israel (Bald et al. 1983; Lawson and Hsu 1996; Lebiush-Mordechai et al. 2014; Li et al. 2013; Loffler et al. 1995; Shang et al. 2014). Moreover, root disease of *Crocus* (a member of the Iridaceae family) was associated with *P. penetrans* and *P. pratensis* complexed with two fungal species, *Stromatinia gladioli* and *Pythium* sp. (Schenk 1970).

This work characterizes potential pathogenic fungal species present in fields used for extensive production of Easter lilies in Oregon and investigates symptom development under combined infection with these fungi and the most common root lesion nematode species, *P. penetrans,* found in those fields. The association of pathogenic fungi with lilies has been characterized and reported mainly for those countries where lilies are of significant economic importance. Identification of eight randomly selected fungal isolates in our survey revealed the presence of one *Rhizoctonia* sp. AG-I isolate, two *F. tricinctum* isolates, and five *F. oxysporum* isolates. Infection of lily roots by the different fungal isolates was observed using single fungal infections in vitro, and the majority of roots showed the development of severe necrotic symptoms by 14 DAI, indicating that each isolate was able to colonize and cause disease in lilies. Early in the infection process, some roots became translucent in appearance rather than being opaque, and showed a lack of turgidity. By 30 DAI, most lily roots and shoots were completely infected with fungus visible over the surface of the plant.

The infection of lily plants by soilborne fungi has been reported as occurring singly or in a complex association of fungal species capable of inducing severe root rot of the roots and consequently diminishing the development and the value of this floral crop. For example, a binucleate *Rhizoctonia* AG-A (syn. *Ceratobasidium* species) and *R. solani* has previously been reported as pathogens on lily (Bald et al. 1983; Lebiush-Mordechai et al. 2014), while *F. tricinctum* has been reported to infect *Lilium davidii* cv. *unicolor* in China, causing stem and root rot (Shang et al. 2014). Similar to our results, Bald et al. (1983) reported that a high proportion of fungal isolates from diseased roots, stems, and bulbs of Easter lily were *F. oxysporum* while Rajmohan et al. (2011) identified six isolates from diseased bulbs as belonging to *Fusarium solani.* Both *F. oxysporum* and *F. solani* were isolated from bulbs of diseased *L. longiflorum* cv. Nellie White plants grown in New Jersey, and *F. solani* was found infecting bulb scales of diseased 'Nellie White' plants that had been grown in the Smith River region between Brookings, OR, and Smith River, CA (Rajmohan et al. 2011). Surprisingly, as Easter lilies used in this study were collected from similar areas of Brookings, OR, none of the eight isolates identified in this study were *F. solani*. The differences found for both studies could be a result of sampling similar geographic areas in different years and seasons or it may reflect different areas of the field. A more comprehensive study involving a larger number of samples collected during different times in similar areas of the field is needed to clearly elucidate the complete range of fungal isolates capable of infecting lilies in these fields.

Although the ITS rDNA sequences of the *F. oxysporum* isolates in our investigation were identical (99 to 100% identity), variations in pathogenicity were observed among them. The isolates also differed in their ability to produce spores on PDA. Nevertheless, the question of whether all the *Fusarium* isolates in our survey belong to one or more pathogenic strains cannot be resolved at this point. Multilocus phylogenetic analysis (Ortu et al. 2015) has been used to define the subspecies status of *F. oxysporum* f. sp. *echeveriae*. Recently, a metagenomic profiling of the rhizosphere associated with healthy and wilted *L. davidii* var. *unicolor* lilies revealed that there were higher levels of *Fusarium*, *Rhizoctonia*, *Verticillium*, *Penicillium*, and *Neonectria* in the rhizosphere of wilted lilies as compared with the rhizosphere of healthy lilies (Shang et al. 2016), although actual infection of healthy lilies with isolates of these fungal species was not performed in that study. Our results appear to follow a similar trend, as the most common fungus isolated from infected lilies collected from the field was *Fusarium*, with *F. oxysporum* most prominent, and a single isolate of *Rhizoctonia*.

Several factors have been reported to contribute to an effective interaction between *P. penetrans* and *Rhizoctonia* in strawberries or *Fusarium* in chickpeas. In wilt-susceptible chickpeas, an accelerated increase in the *P. penetrans* population was observed that exceeded the normal rate of population growth, and this may result from conditions that are beneficial to growth of nematodes within the cortical cells resulting from *Fusarium* infection (Castillo et al. 1998). *Pratylenchus penetrans* penetrates roots of strawberry plants and destroys or weakens cortex cells as it travels intracellularly throughout the length of the root making the root cells more susceptible to infection by *R. fragariae* (LaMondia and Cowles 2005). Larvae of *Maladera*

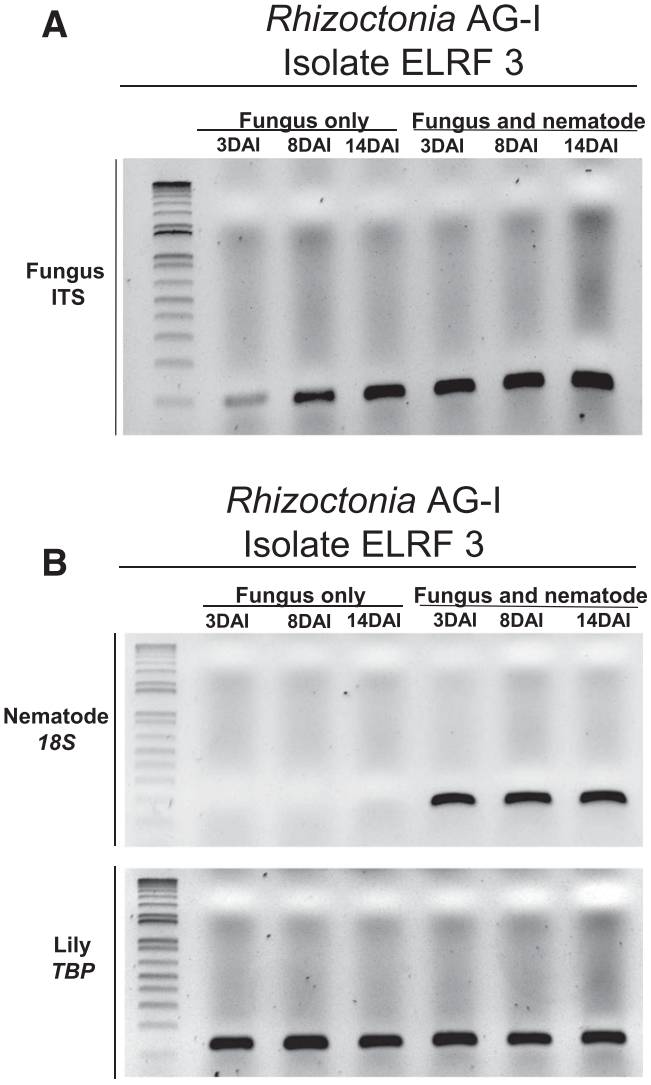

Fig. 4. A, PCR analysis of the genomic DNA isolated from *L. longiflorum* roots 3, 8, and 14 DAI with *Rhizoctonia* AG-I isolate ELRF 3 and either with (+) or without (−) three days prior inoculation with *P. penetrans*. Primers amplified the *Rhizoctonia* ITS region. **B,** Reverse transcription PCR analysis of RNA isolated from *L. longiflorum* roots 3, 8, and 14 days after inoculation with *Rhizoctonia* AG-I isolate ELRF 3 and with (+) or without (-) prior inoculation with *P. penetrans*. Primers amplified a region of the 18S rRNA of *P. penetrans*. Primers to P450-like TBD protein were used to verify extraction of RNA from the lily roots and cDNA synthesis.

castanea, the Asiatic garden beetle, also feed on strawberry roots, but unlike *P. penetrans*, their feeding did not increase disease severity caused by *R. fragariae*, possibly because after feeding, there was an insufficient amount of root tissue remaining to nourish the *Rhizoctonia* (LaMondia and Cowles 2005). In our studies, prior infection with the nematode generally resulted in a more advanced development of disease symptoms and a more rapid growth of the fungus in the roots.

In the particular case of field-grown Easter lilies in Oregon, *P. penetrans* is considered one of the most difficult pathogens to control. *Pratylenchus penetrans* is also a major problem for raspberries grown in the Pacific Northwest, where 92% of processed raspberries are grown (Zasada et al. 2015). Zasada et al. (2015) found that soil texture is a critical factor affecting the damage to crops caused by *P. penetrans*. There is more damage from *P. penetrans* when raspberries are grown in a sandy soil as compared with a finer textured soil, possibly because the soil environment influences movement, fecundity, and survival of the nematode. In the United States, Easter lilies are primarily grown on the coast of the Pacific Northwest, where the climate allows them to flourish, although fields in this location have the Knappa-heavy variant type of soil that consists of a 13-cm layer of silty clay loam (Lissner 1977; USDA Soil Conservation Service 1970) where *P. penetrans* also flourishes.

This study identified, growing in a field in Oregon, a complex network of fungal isolates that are able to infect lily roots. To our knowledge, this is the first report that *Rhizoctonia* sp. AG-I infects lilies and the first report of *F. tricinctum* infecting lilies in the United States. Infection of Easter lilies by all eight fungal isolates appeared to be accelerated when there had been prior inoculation with *P. penetrans*. Both pathogen identity and their contribution to infection are factors necessary to consider in a pest management strategy (Borer et al. 2016; Lamichhane and Venturi 2015). The importance of controlling both the fungal pathogens and *P. penetrans* when growing lilies in the field has been clearly demonstrated. In future studies, it would be useful to conduct more detailed studies with lilies growing in the greenhouse to determine the extent of decrease in biomass of the roots and shoots that is caused by the fungal pathogen and/or *P. penetrans*, and how this ultimately affects flower performance. Investigations should also focus on finding the primary and secondary pathogens of lily root rot and determine temporal and spatial succession of pathogens that lead to maximum root damage. It also remains to be seen if specific pathogen combinations interact in an additive, synergistic, or antagonistic way to enhance or reduce the disease syndrome. The outcome of such findings should benefit the formulation of the most effective disease management strategy in terms of selection and application of pesticides.

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