RESEARCH

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Characterization of the Fungal Community Associated with Root, Crown, and Vascular Symptoms in an Undiagnosed Yield Decline of Winter Squash

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Accepted for publication 7 November 2019.

ABSTRACT

Winter squash (*Cucurbita maxima*) is produced in Oregon's Willamette Valley for edible seeds, processing, and fresh markets. Recently, prominent cultivar Golden Delicious has experienced significant yield losses due to a soilborne disease. Symptoms include stunting, root and crown rot, vascular discoloration, and late-season vine collapse. To identify potential causal pathogens, 64 fields were surveyed during 2014 to 2016 to characterize the fungal community associated with surface-disinfested root, crown, and stem tissue of diseased and healthy squash. Over 10,000 fungal isolates were identified morphologically, and 1,783 isolates were identified to species by sequencing of internal transcribed spacer and translation elongation factor 1 alpha genomic regions. Fungal communities were analyzed for association with the presence or absence of field symptoms using multivariate community analyses (indicator species analysis, multiresponse permutation procedure, and nonmetric multidimensional scaling).

Although no fungal species were consistently associated with disease, five species were consistently isolated from plants regardless of the presence of symptoms and were capable of causing disease in a greenhouse pathogenicity trial: *Fusarium oxysporum*, *F. solani*, *F. culmorum*, *Plectosphaerella cucumerina*, and *Setophoma terrestris*. Results from community analyses confirmed that some fungi were more common in specific tissues (e.g., *P. cucumerina* in stems and crown, *F. solani* in roots and crown). Symptom severity tended to be greater in fields with a prior history of squash production, although a few fields with no prior history of squash had above average symptom ratings. The results from this study suggest that the five most common fungi may take on greater disease significance when they co-occur in a host.

Keywords: agriculture, ecology, plant pathology

Winter squash (*Cucurbita maxima*) is grown in Oregon's Willamette Valley for edible seed production, frozen food processing, and fresh market production, which together comprise an annual 7-million-dollar industry (Taylor and Losh 2018). Over 2 decades, industry personnel and extension agents have reported an emerging soilborne disease problem in winter squash with symptoms that include root and crown rot, vascular discoloration, stunting, and reduced fruit size and seed yield. In extreme cases, a

late-season vine collapse has caused crop failures. National Agricultural Statistical Service surveys (USDA/NASS QuickStats Ad-hoc Query Tool at <https://quickstats.nass.usda.gov/>) reported yields of greater than 15 tons per acre in 2000 and 2001, but an average of only 9 tons per acre from 2002 to 2014. In 2015, production in mostly new regions of the valley had yields over 15 tons per acre, but these regions are at increasing distances from the processing facilities. Research that could potentially ameliorate disease-induced production losses in winter squash is hampered by a lack of understanding of causal agents of the yield decline problem.

Soilborne fungal pathogens have plagued cucurbit production in different regions of the world. In some cases, cucurbit diseases are attributable to a single pathogen, while in others, more complex pathogen interactions exist. Races of *Fusarium solani* f. sp. *cucurbitae* have been identified as the causal agent of cortical stem rot and fruit rot in pumpkin and winter squash species in California and Pennsylvania (Mehl and Epstein 2007; Samac and Leong 1989). *F. oxysporum* f. sp. *melonis* has been identified as the causal agent of melon collapse and root rot in California and New

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Funding: Partial funding was provided by USDA NIFA Western SARE project SW15-021.

*The e-Xtra logo stands for "electronic extra" and indicates that three supplementary appendixes are published online.

The author(s) declare no conflict of interest.

York (Gwynne et al. 1997; Zuniga et al. 1997). In California and Texas, both *F. oxysporum* and *F. solani* have been found to be causal agents of fruit and root rots of muskmelon species (Champaco et al. 1993; Mertely et al. 1991). And these same *Fusarium* species have been found in complex with other fungal pathogens such as *Acremonium cucurbitacearum*, *Monosporascus cannonballus*, and *Pythium* spp. causing melon collapse (Aegerter et al. 2000; Martyn and Miller 1996). In Japan, winter squash (*C. moschata*) has been damaged by pink root rot caused by *Setophoma terrestris* (Ikeda et al. 2012). In Europe, melon vine collapse caused yield reductions and was attributed to *A. cucurbitacearum*, *M. cannonballus*, *Plectosphaerella cucumerina*, and *Rhizopycnis vagum* (Carlucci et al. 2012; Chilosi et al. 2008). However, in Italy and Spain, crown rot and vine collapse of zucchini and winter squash has been attributed to *F. solani* and/or *P. cucumerina* (García-Jiménez et al. 1997; Vitale et al. 2007). Commonly, these research reports describe symptoms similar to those observed in Oregon-grown winter squash, and frequently more than one fungal pathogen is associated with the symptoms, which is indicative of a pathogen complex (Fitt et al. 2006; Lamichhane and Venturi 2015; Le May et al. 2009).

Winter squash, though an important Oregon crop, has not received the research attention of other cucurbit species. Of critical importance is to determine whether the disease is caused by a single or multiple causal agents and which cultivation practices are associated with disease severity. A 3-year survey of commercial squash fields was conducted to obtain information on the culturable fungi associated with symptoms in roots, crowns, and vascular stem tissues of squash plants. Multivariate statistics, commonly used in community ecology and diversity studies (McCune and Mefford 1999), were employed to evaluate the pool of potential causal agents with disease symptoms and to identify grower practices that may be associated with disease incidence. Specific questions that were asked to gain an understanding of this pathosystem included the following: (i) Is there a consistent set of fungal species associated with symptoms of vine collapse and root rot of winter squash? (ii) Are these fungal species unique to *C. maxima*, or do other commercially grown cucurbit species also harbor these fungi? (iii) Are the same fungi found in the stem, crown, and root tissue? And (iv) is there a difference between species associated with symptoms in plants grown under a processed vegetable rotation, a fresh market rotation, a mint rotation, a fallow rotation, or a grass seed rotation? Ultimately, the survey results were expected to create a shortlist of potential pathogenic

agents for further evaluation in pathogenicity tests to fulfill Koch's postulates.

MATERIALS AND METHODS

Two approaches were used to survey squash fields. In 2014 and 2015, nonrandom samples of *C. maxima*, *C. moschata*, *C. pepo*, and *Cucumis sativus* were made to characterize the fungal communities associated with symptomatic and asymptomatic plants and to identify the potential causal agents of disease. In 2016, the scope was focused on *C. maxima* only and involved plants sampled randomly to examine disease symptom occurrence and severity in relation to the fungi present in squash tissues and to crop rotation history. Throughout, the survey approaches were kept separate by year.

Field survey to characterize fungal community associated with Willamette Valley cucurbits (2014–2015). Cucurbit plants were collected from 12 commercial squash fields in 2014 and seven fields in 2015 located in Benton, Lane, Linn, Marion, and Yamhill counties in Oregon. Most fields (80%) were planted for processing, whereas the others were planted for local fresh markets. Processing fields ranged from 5.3 to 32.4 ha and fresh market fields were 1.2 to 3.2 ha. From both processing and fresh market fields, four different cucurbit species were sampled: *C. maxima*, *C. moschata*, *C. pepo*, and *Cucumis sativus* (Table 1). From each field, an average of 20 plants were collected from early July to early September (Table 1); the number of plants collected ranged from 4 to 30 with more samples from fields with symptoms, including wilting, stunting, and/or chlorosis. Plants were collected throughout the fields to include both asymptomatic and symptomatic plants. Plants were dug to preserve as many roots as possible, and vines were trimmed to a length of 0.3 to 0.6 m proximal to the crown.

After collection, plants were washed to remove soil and then assessed for symptoms by cutting open the roots, crown (5-cm section of stem immediately adjacent to the roots), and stem (>5 cm from the roots, but below the first branch). In 2014, plants were rated symptomatic or asymptomatic depending on the presence of discoloration or rot. In 2015, a symptom severity rating scale was developed where plants were rated from 1 to 4 for roots, crowns, or stem vascular tissue: 1, no yellowing/discoloration; 2, localized yellow to orange discoloration; 3, localized orange to brown discoloration; and 4, complete discoloration. Plants with two tissue ratings of 2 or higher or at least one rating of 3 or 4 were considered

TABLE 1
Summary of winter squash (*Cucurbita* spp.) fields surveyed from Oregon's Willamette Valley from 2014 to 2016 growing seasons to characterize fungal communities associated with healthy and diseased squash tissues

Year	Number of fields	Field history of cucurbit production			Number of plants collected ^x	Healthy plants ^y	Diseased plants ^z	Number of plants of each cucurbit species				Number of isolates morphologically identified	Number of isolates molecularly identified
		History of disease ^v	Never planted in cucurbits ^w	Symptom development in season				<i>C. maxima</i>	<i>Cucumis sativus</i>	<i>C. moschata</i>	<i>C. pepo</i>		
2014	12	9	3	10	282	101	181	200	20	27	35	4,244	1,399
2015	7	5	0	6	94	55	39	34	11	42	7	2,050	121
2016	49	10	25	43	1,632	923	611	1,632	0	0	0	4,497	263

^v Fields with a history of disease according to crop consultants.

^w Never planted in cucurbits according to 5-year rotation history obtained from growers and crop consultants.

^x Number of plants collected in each growing season. In 2014, 83 plants were collected from fresh market fields and 199 plants were collected from processing crop fields. In 2015, 33 plants were collected from fresh market fields and 61 plants were collected from processing crop fields.

^y In 2014, plants were classified as healthy if they did not exhibit any stem discoloration, crown rot, or root rot symptoms. In 2015 and 2016, plants with all disease severity ratings of 1 or only one rating of 2 were classified as healthy.

^z In 2014, plants were classified as diseased if they showed any stem discoloration, crown rot, or root rot symptoms. In 2015 and 2016, plants with at least two disease severity ratings of 2 or at least one rating of 3 or 4 were classified as diseased.

symptomatic, and plants with all 1 ratings or only one rating of 2 were considered asymptomatic.

Fungal isolation and identification. Fungi were isolated from the vascular tissue of the roots, crowns, and stems. In a laminar flow hood, 1- to 2-mm cross sections of each tissue were cut with a flamed knife and then quartered into 1- to 2-mm wedges that were disinfested in a 1.13% NaOCl solution for 2 min, rinsed once in sterile water for 30 s, and dry blotted on a paper towel. Four pieces from each tissue type were placed equidistantly apart on plates of 1/8th strength potato dextrose agar (PDA; 4.875 g of PDA, 12 g of Bacto agar, and 1 liter of water) (Difco, Franklin Lakes, NJ) in 9-cm Petri plates amended with chlortetracycline at 20 µg/ml and streptomycin at 50 µg/ml (all subsequent media utilized in this study were amended with these antibiotics).

Plates were incubated in crisper boxes at 22 to 25°C for 5 to 7 days in a 12 h light/dark cycle. Following incubation, plates with fungi were photographed, and fungi present were identified to species morphologically (Dugan 2006; Leslie and Summerell 2006) (data on morphological identities were reconfirmed in 2018 by review of the photographs). A representative subset of visually identified fungi (two and six per plant) were transferred to fresh 1/8th PDA to prepare for molecular identification. To ensure pure cultures, all isolates were single-spored following the *Fusarium* Laboratory Manual protocol (Leslie and Summerell 2006). Cultures were maintained on Spezieller Nährstoffarmer agar (SNA). Fungi that did not produce spores in culture were examined under a dissecting microscope for individual growing hyphal tips that were extracted once and maintained on SNA. These non-spore-forming cultures represented approximately 2% of the total pure cultures.

In total, 1,520 pure (single spore or hyphal tip) cultures that were representative of the diversity of fungal cultures identified morphologically were subjected to DNA sequencing and identification. In 2014, DNA was extracted with DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) (Griffin et al. 2002), and in 2015, with InstaGene Matrix (Bio-Rad Laboratories, Hercules, CA) (Ciardo et al. 2006). Extracted DNA was subjected to PCR of the internal transcribed spacer 1 alpha (*ITS1*) region (White et al. 1990) and translation elongation factor 1 alpha (*EF1α*) gene region (Leslie and Summerell 2006). PCR amplicons were cleaned with ExoSapIT (Affymetrix, Santa Clara, CA) and submitted to the Oregon State University Center for Genome Research and Biocomputing (CGRB) for sequencing. Sequences were entered into the NCBI BLAST and FusariumID databases for identification (accession numbers MN521703 to MN521808, MN521834 to MN523210, and MN553564 to MN553577). A sequence match of greater than 97% similarity was considered indicative of a species in this study. A sequence match less than 97% similarity was categorized into genus only.

Statistical analysis of 2014–2015 survey data. Data on isolate identity were subjected to multivariate statistical analyses to characterize associations among fungal species and the host tissues from which they were recovered. Identities based on morphology were analyzed separately from those based on sequence analysis. Morphological identifications included a larger number of fungal isolates with a relatively narrower scope of identification due to limitations of morphological identification methods, and the molecular identifications included a smaller number of isolates recovered from a fewer number of plants but had a broader scope of identification (Supplementary Appendix S1).

Nonmetric multidimensional scaling (NMS or NMDS), an ordination analysis, was used to evaluate whether the fungal community differed between symptomatic and healthy plants, cucurbit species sampled, plant tissue type, and processed vegetable versus fresh market production fields. This method does not assume linear relationships among variables and allows for comparison of categorical variables

(Kruskal 1964; Mather 1976; McCune and Grace 2002). Morphological identifications were entered into a primary species matrix that contained a row for each plant collected (376) and a column for fungal species (15) identified through visual evaluations in 2014 and 2015. Counts of each fungus per plant were entered into the corresponding matrix cell. A secondary trait matrix was created with the same number of rows (plants) and 14 columns of grouping variables: six categorical (field, cultivar, year, diseased [yes or no], cucurbit species, and crop purpose) and eight quantitative variables (0 or 1 response): symptomatic, asymptomatic, *C. maxima*, *C. moschata*, *C. pepo*, *Cucumis sativus*, processing, or fresh market. Primary and secondary matrices were similarly created for each specific tissue of isolate recovery (831 rows); in this case, the secondary matrix had the previous 14 columns plus an additional 4 columns for tissue type: one categorical variable (tissue) and three quantitative variables (stem, crown, and root). Analogous matrices for molecular identifications were created and were analyzed separately from the morphological data. The molecular primary matrix had 337 plant rows and 86 columns of unique fungal species; the matrix had 449 tissue rows and 86 fungal species. Secondary matrices were the same as described above.

After creating matrices, outlier analysis utilizing Sørensen distance measurements was conducted in PC-ORD (Version 7, MjM Software, Gleneden Beach, OR) to remove plants with fungal communities greater than two standard deviations from the grand mean, as such extreme statistical outliers may obscure important biological associations in these kinds of multivariate analyses (McCune and Grace 2002). From the morphological dataset, 39 plant rows and 185 tissue rows were removed. From the molecular dataset, 67 plant rows and 5 tissue rows were removed. Additionally, in the molecular dataset, those fungal species that only had one or two total counts were removed (51 species). The NMS analysis was run in PC-ORD using the “medium” autopilot settings in Sørensen distance (50 randomized runs and 50 real runs, penalizing for ties, which provides a more conservative result). Following ordination construction based on the primary matrix, variables from the secondary matrix were overlaid to identify groupings related to the fungal community.

To estimate the strength of association between a potential causal agent and a category of interest (diseased or healthy, cucurbit species, farm type, or tissue type), indicator species analysis (ISA) was used. With ISA, each species receives an indicator value that is the product of consistent presence in an environmental group and frequency of occurrence in that group; i.e., a species has a perfect indicator (value = 100) when it is always present in a group and exclusive to that group (Dufrene and Legendre 1997; McCune and Grace 2002). Consistent presence is calculated as total occurrences of species in a group divided by total occurrences in all groups, and species frequency of occurrence is the proportion of sample units in each group that contain that species. The same datasets described for NMS were used for ISA and utilized the same outlier analysis. ISA was run in PC-ORD with Monte Carlo estimation from 4,999 random runs.

Pathogenicity testing. For fungal species isolated frequently, greenhouse evaluations of pathogenicity were conducted during the winter of 2016–17. Five spore-forming fungi were selected for this evaluation: *F. oxysporum*, *F. solani*, *F. culmorum*, *Plectosphaerella cucumerina*, and *Setophoma terrestris*. Six individual isolates of each fungal species collected from different fields were cultured individually in 175 ml of potato dextrose broth for 7 days at 22°C on a shaker at 400 rpm. Inocula were prepared following methods of Punja and Parker (2000). Conidia concentrations were adjusted to between 10⁵ and 10⁷ spores per ml with aid of a hemocytometer (confirmed by dilution plating onto PDA). In a greenhouse, 3-week-old seedlings of *C. maxima* ‘Golden Delicious’ grown in Sunshine Professional Mix 4 (Sun Gro Horticulture, Agawam, MA) were uprooted and washed. Roots were trimmed to 5-cm in length and

placed into a spore suspension (hand shaken to resuspend spores) or broth-only control for 3 min. Inoculated seedlings, three per isolate and 18 per fungal species or control, were replanted in 3.8-liter pots and arranged in a completely randomized design on benches. The greenhouse was maintained at 27°C with a 12-h light/dark cycle for 8 weeks. As plants exhibited symptoms or after 8 weeks of growth, they were destructively harvested. Harvested plants were washed, rated for symptoms in root, crown, and stem tissues on the 1 to 4 rating scale described above. Each of the tissues also were cut into small pieces, surface disinfested, and plated onto PDA to attempt to reisolate inoculated fungi. Recovered isolates were identified based

on morphology and *ITS* sequences. This experiment was conducted once.

Symptom severity ratings were analyzed using multiresponse permutation procedure (MRPP), which is a test for group membership based on nonparametric multivariate data (McCune and Grace 2002). Each of the 108 plants treated with one of the five fungal species or a control were entered into a primary matrix that had a column for each tissue rating. A secondary matrix had the same list of plants and two categorical variables: fungal species and isolate. MRPP utilized the two matrices to calculate the multivariate distance similarity among samples within each group and to

TABLE 2
Fungal species isolated from *Cucurbita maxima*, *C. moschata*, *C. pepo*, and *Cucumis sativus* plants collected from fields in Oregon's Willamette Valley during the summers of 2014 and 2015 summarized by method of isolate identification^v

Data set and year	Species	Total count	Total percent	Number from		Number of plants of each cucurbit species ^w				Processing squash	Number from			
				Diseased plants ^x	Healthy plants ^y	<i>C. maxima</i>	<i>C. moschata</i>	<i>Cucumis sativus</i>	<i>C. pepo</i>		Fresh market squash	Stem	Crown	Root
Morphological														
2014	<i>Plectosphaerella cucumerina</i>	719	17	495	224	461	68	62	128	307	412	196	382	147
	<i>Fusarium oxysporum</i>	887	21	646	241	625	118	57	87	684	203	62	431	389
	<i>F. solani</i>	532	13	417	115	245	35	89	163	148	384	37	231	266
	<i>F. culmorum</i>	71	2	40	31	46	4	2	19	43	28	22	24	25
	Other <i>Fusarium</i> sp.	348	8	245	103	200	82	23	43	208	140	100	139	100
	<i>Setophoma terrestris</i>	152	4	114	38	103	37	0	12	133	19	5	101	46
	Other fungi	1,535	36	1,109	426	998	196	87	254	775	760	458	489	593
	Total	4,244	100	3,066	1,178	2,678	540	320	706	2,298	1,946	880	1,797	1,566
2015	<i>P. cucumerina</i>	536	26	260	276	143	296	35	62	275	261	195	204	137
	<i>F. oxysporum</i>	465	23	180	285	219	165	60	21	368	97	52	210	203
	<i>F. solani</i>	282	14	127	155	117	105	38	22	184	98	64	124	94
	<i>F. culmorum</i>	42	2	20	22	9	23	5	5	29	13	14	15	13
	Other <i>Fusarium</i> sp.	210	10	72	138	100	87	7	16	175	35	61	90	59
	<i>S. terrestris</i>	81	4	26	55	43	38	0	0	81	0	4	56	21
	Other fungi	434	21	170	264	136	212	56	30	234	200	163	112	159
	Total	2,050	100	855	1,195	767	926	201	156	1,346	704	553	811	686
Molecular ^z														
2014	<i>Plectosphaerella cucumerina</i>	265	19	92	173	184	1	20	60	71	194	65	157	43
	<i>Fusarium oxysporum</i>	351	25	135	216	253	10	39	49	213	138	24	194	133
	<i>F. solani</i>	174	12	54	120	85	3	43	43	41	133	17	68	89
	<i>F. culmorum</i>	19	1	7	12	15	1	0	3	12	7	1	7	11
	Other <i>Fusarium</i> sp.	180	13	79	101	120	7	21	31	76	104	50	90	40
	<i>Setophoma terrestris</i>	58	4	27	31	46	6	0	6	50	8	7	37	14
	Other fungi	352	26	146	206	242	7	44	60	147	205	107	133	112
	Total	1,399	100	540	859	945	35	167	252	610	789	271	686	442
2015	<i>P. cucumerina</i>	40	33	31	9	10	12	4	11	8	32	19	16	5
	<i>F. oxysporum</i>	45	37	32	13	19	10	8	2	27	18	1	23	21
	<i>F. solani</i>	12	10	9	3	7	2	2	0	8	4	1	7	4
	<i>F. culmorum</i>	2	2	2	0	0	0	1	0	0	2	1	1	0
	Other <i>Fusarium</i> sp.	13	11	9	4	7	3	1	0	10	3	2	8	3
	<i>S. terrestris</i>	2	2	2	0	1	1	0	0	2	0	0	1	1
	Other fungi	7	5	4	3	2	3	2	0	2	4	3	2	2
	Total	121	100	89	32	46	31	18	13	57	64	27	58	36

^v Supplementary Appendix S1 provides details of all other fungi identified.

^w Supplementary Appendix S2 provides details of all cucurbit cultivars sampled.

^x In 2014, plants were classified as diseased if they showed any stem discoloration, crown rot, or root rot symptoms. In 2015 and 2016, plants with at least two disease severity ratings of 2 or at least one rating of 3 or 4 were classified as diseased.

^y In 2014, plants were classified as healthy if they did not exhibit any stem discoloration, crown rot, or root rot symptoms. In 2015 and 2016, plants with all disease severity ratings of 1 or only one rating of 2 were classified as healthy.

^z Isolates identified through sequencing of *ITS* and *EF1α* genes.

compare the similarities to the other groups with a chance-corrected t statistic (McCune and Grace 2002; Mielke et al. 1981). MRPP was used to assess whether the fungal species treatment was associated with differences in symptom severity compared with the broth-only control treated plants. The level of significance for P values was adjusted for 15 comparisons using a Bonferroni correction and set to a new significance level of 0.0033 (Shaffer 1995; Steel et al. 1997).

2016 field survey of commercially grown *C. maxima* ‘Golden Delicious’ plants. A third year of survey was conducted in 2016 with a redesigned purpose. The objective was to sample plants from multiple, unbiased points within fields to obtain an estimate of how frequently potential pathogens identified from the previous surveys could be detected, and to assess if rotation history was related to observed symptom severity and frequency of fungal species recovery. Forty-nine fields of *C. maxima* ‘Golden Delicious’ located in Benton, Lane, Linn, Marion, and Yamhill counties in Oregon were selected for sampling with assistance from local crop advisers. Field sizes ranged from 3 to 138 ha with most fields between 10 and 30 ha. All fields were sampled near crop maturity (between 2 and 11 September). For every 10 ha in a field, a random point was generated using PlotHound software (SilviaTerra, San Francisco, CA), and at each point, 20 plants were collected at 7.6-m intervals (i.e., 152-m transect). For smaller fields (3 to 8 ha), two points were obtained from PlotHound and a 10-plant sample was collected from each point, totaling 20 plants. One exceptionally large field (138 ha) was sampled at only six points. As in previous seasons, plants were collected to preserve roots, and vines were trimmed to 0.3 to 0.6 m above the crown.

Plants were washed and evaluated for symptoms with the 1 to 4 rating scale developed in 2015. Three representative plants per 20-plant sample and two plants per 10-plant sample were processed for fungal isolation as described previously. After incubation on 1/8th

PDA for 1 week, isolates were identified morphologically and photographed as described previously (photographic data were re-evaluated in 2018 to confirm morphological identification). A subset of morphologically identified fungi were single-spored and a total of 1,571 isolates were selected for DNA sequencing and identification. DNA extraction using InstaGene Matrix and PCR of the *ITS* and *EF1 α* gene regions were conducted as previously described (Accession numbers MN521460 to MN521696).

Five-year field cropping histories were obtained from advisors and growers. Fields were assigned a primary rotation crop based on the previous 5 years. Primary rotation crops were defined as grass seed (>2 years in grass seed production, 19 fields), processed vegetables (≥ 2 years in vegetable production, either snap bean, sweet corn, beet, peas, spinach, broccoli, cauliflower, or winter squash, 13 fields), mint (≥ 2 years in mint production, 3 fields), fallow (≥ 2 years in fallow or no production, 1 field), or unknown (from 13 fields from which rotation histories were not obtained). A count of the number of years previously planted in winter squash was recorded for each field (0, 1, or 2 years out of 5). Fields with unknown histories were excluded from further analyses, leaving 36 fields with known rotation history.

Growers (mostly in the north valley) with no history of squash production have been growing squash on new ground. For this reason, all fields were assigned a north or south valley designation, to assess if there was a difference in symptom severity and/or species composition between those groups. Fields were labeled north if they were located above 45° latitude and south if below this latitude.

Statistical analysis of 2016 survey data. The relationship between symptom severity and crop rotation history was examined using MRPP. Each of the 1,148 plants collected in 2016 with rotation histories was entered into a primary matrix that had a column for each tissue rating. A secondary matrix had the same list of plants and four categorical variables: field, location, primary previous crop, and years previously in squash. MRPP was used to assess whether field location in the valley (south or north), primary previous crop (last 5 years), and years previously planted in squash were associated with differences in symptom severity. For example, the similarity of tissue ratings from southern fields, which are nearer to processing facilities, were compared with plants from northern fields, where squash is a more recent crop.

Symptom ratings were visualized by tissue, years in squash previously, primary rotation crop, and location in the valley using graphing methods in RStudio (version 1.1.456, RStudio Inc., Boston, MA). Histograms of symptom ratings were constructed for each host tissue to compare categories of primary previous crop and of years planted previously in squash. After review of the histograms, a χ^2 test of independence was conducted to test similarity of isolation frequency of selected fungal species from plant tissues rated high (symptom ratings of 3 or 4) compared with those rated low (symptom ratings of 1 or 2) to determine if specific species incidence differed among these categories (McDonald 2014).

RESULTS

Condition of sampled plants in 2014 and 2015. Of the 12 fields sampled in 2014, nine fields had a history of disease and 10 showed visible wilting symptoms during the growing season (Table 1). Sixty-four percent of plants collected were symptomatic, exhibiting primary symptoms of vascular discoloration, crown rot, root rot or a combination of the three. Sixty percent of plants from processing fields were considered symptomatic, while 73% of plants from fresh market fields were symptomatic. In 2014, 4,244 isolates were identified morphologically, and 1,399 were sequenced and identified through sequence similarity in GenBank (Table 2).

Fig. 1. Nonmetric multidimensional scaling ordination of the 35 fungal species isolated from surface disinfested tissues categorized by cucurbit crop purpose. Sampled plants were collected in surveys of commercial fields during the 2014 and 2015 growing seasons (Table 1). Red triangles represent fungal communities associated with individual plants from fresh market fields. Green triangles represent fungal communities associated with individual plants from processed vegetable fields. Blue dots show the centroid of fungal species in dimensional space with labels provided for five of the most commonly isolated fungi. Axis 1 explains 0.468 variance and axis 2 explains 0.143 variance.

Of the seven fields sampled in 2015, six showed wilt symptoms during the growing season, and five had a history of cucurbit production in the recent past (Table 1). Forty-one percent of collected plants exhibited symptoms. Plants collected from processing vegetable fields accounted for 64% of all plants in 2015, and of those plants, 34% exhibited disease symptoms. From the 36% of plants collected in fresh market fields, 52% exhibited disease symptoms. In 2015, with the newly developed rating scale, all plants had a mean stem discoloration rating of 1.97 out of 4, a mean crown rating of 2.20 out of 4, and a mean root rating of 2.38 out of 4. Throughout the growing season, 2,050 isolates were morphologically identified, and a representative 121 isolates were subjected to DNA extraction and sequencing for molecular identification (Table 2).

Characterization of fungal communities in squash tissues. A total of 6,294 isolates were collected from 376 plants in the 2014 and 2015 growing seasons (Table 1). Of these isolates, 2,361 (56%) in 2014 and 1,405 (70%) in 2015 were morphologically identified as one of three *Fusarium* species (*F. oxysporum*, *F. solani*, and *F. culmorum*), *P. cucumerina*, or *S. terrestris* (Table 2). In both years, the fewest number of isolates were cultured from stems (23%), but from those stem tissues, *P. cucumerina* was recovered most frequently (27% of all stem cultures, Table 2). Ten other fungal species were identified morphologically, which included *Colletotrichum coccodes*, *Diaporthe sclerotoides*, *Epicoccum nigrum*, and *Mucorales*-type fungi (Supplementary Appendix S1). In the subset of 1,520 isolates subjected to PCR analysis of the *ITS* and *EF1 α* genomic regions, 86 unique fungal species were identified, and of those identified, 26% were *F. oxysporum*, 20% were *P. cucumerina*, 12% were *F. solani*, 1% were *F. culmorum*, and 4% were *S. terrestris* (Table 2), with 81 other species accounting for the remaining 37% of cultures (Supplementary Appendix S1; accession numbers MN521703 to MN521808, MN521834 to MN523210, and MN553564 to MN553577).

Fungal communities are the species that were cultured from winter squash and cucurbits in this study as grouped by one of the four environmental categories defined previously: symptomatic versus asymptomatic, cucurbit species, farm/rotation type, and tissue type. With both the morphological and molecular identifications, NMS ordinations did not suggest that fungal communities associated with symptomatic and healthy plants were strongly differentiated (data not shown). Similarly, fungal communities from the four sampled cucurbit species were not clearly differentiated by the host from which they were recovered (data not shown). *P. cucumerina* and *F. solani*, in both the morphological and molecular datasets were more strongly associated with cucurbit plants from fresh market fields compared with plants grown for processing (Fig. 1). Ordinations of specific host tissues also showed that *F. oxysporum*, *F. solani*, and *S. terrestris* were associated with crowns and roots, whereas *P. cucumerina* was associated with stems (data not shown).

Indicator species analysis found similar patterns to NMS, as well as some more specific comparisons among variables. For ISA, several fungi (*P. cucumerina*, *F. oxysporum*, and *F. solani*) had high indicator values relative to the other species in the matrices (36.6, 47.3, and 35, respectively, for morphological data, and 14.4, 34.3, and 14.4, respectively, for molecular data; Table 3). These indicator values, however, were not statistically associated with either diseased or healthy plants ($P > 0.10$) (Table 3). With regard to cucurbit species, in the molecular dataset, *F. solani* and *P. cucumerina* were significant indicators ($P < 0.04$) of *Cucumis sativus* and *C. pepo*, respectively; these hosts were collected nearly exclusively from fresh market fields. In the morphological dataset, *P. cucumerina* and *S. terrestris* were both identified as significant indicators ($P < 0.01$) of *C. moschata*. *F. solani* was a significant indicator of *C. pepo* ($P < 0.01$). *P. cucumerina* and *F. solani* were identified as significant indicators ($P < 0.01$) of fresh market crops in both data sets and NMS (Table 3, NMS data not shown). In the morphological dataset, *F. oxysporum* and

TABLE 3
Indicator species analysis for selected fungi isolated during the 2014 and 2015 growing seasons from *Cucurbita maxima*, *C. moschata*, *C. pepo*, and *Cucumis sativus* plants collected in the Willamette Valley of Oregon

Data set ^w	Diseased versus healthy			Cucurbit species			Crop purpose			Host tissue		
	Variable ^x	Observed indicator value ^y	P value ^z	Variable	Observed indicator value	P value	Variable	Observed indicator value	P value	Variable	Observed indicator value	P value
Morphological												
<i>Plectosphaerella cucumerina</i>	Diseased	36.6	0.94	<i>C. moschata</i>	30.2	<0.01	Fresh market	63.2	<0.01	Stem	33.8	<0.01
<i>Fusarium oxysporum</i>	Healthy	47.3	0.61	<i>C. moschata</i>	27.5	0.28	Processing	62.4	<0.01	Crown	34.7	<0.01
<i>F. solani</i>	Diseased	35	0.20	<i>C. pepo</i>	29.9	<0.01	Fresh market	60.4	<0.01	Crown	21.9	<0.01
<i>F. culmorum</i>	Healthy	12.3	0.40	<i>C. pepo</i>	12.8	0.07	Fresh market	12.6	0.36	Stem	5.6	0.19
<i>Setophoma terrestris</i>	Healthy	15.6	0.32	<i>C. moschata</i>	24.1	<0.01	Processing	27.9	<0.01	Crown	16.5	<0.01
Molecular												
<i>P. cucumerina</i>	Diseased	14.4	0.99	<i>C. pepo</i>	29	<0.01	Fresh market	48.2	<0.01	Stem	23.8	<0.01
<i>F. oxysporum</i>	Healthy	34.3	0.16	<i>C. maxima</i>	18.2	0.61	Fresh market	36.8	0.04	Crown	29.1	<0.01
<i>F. solani</i>	Healthy	14.4	0.40	<i>Cucumis sativus</i>	16.3	0.04	Fresh market	30	<0.01	Crown	9.7	0.20
<i>F. culmorum</i>	Healthy	5.6	0.03	<i>C. pepo</i>	1.7	0.95	Fresh market	2.8	0.75	Crown	0.9	0.99
<i>S. terrestris</i>	Healthy	5.7	0.12	<i>C. pepo</i>	3.5	0.61	Processing	4.3	0.42	Crown	4	0.08

^w Data set used in indicator species analysis: molecularly identified fungi, or morphologically identified fungi.

^x For each fungal species, the variable with the largest observed indicator value from the categorical group.

^y Observed indicator value based on the relative frequency and abundance of an isolate in a specific group, out of 100.

^z P value from a Monte Carlo test of significance for observed indicator value compared with that expected at random. Bolded values indicate statistically significant P values of ≤ 0.05 .

S. terrestris were significant indicators of processing crops. As with NMS, in both data sets, *F. oxysporum* was identified as a significant indicator of crown tissue and *P. cucumerina* was identified as a significant indicator of stem tissue ($P < 0.01$; Table 3). In the morphological data, *F. solani* and *S. terrestris* were also significant

indicators of crown tissue ($P < 0.01$; Table 3), which was consistent with NMS data.

Pathogenicity of selected fungal species. Inoculation of greenhouse-grown *C. maxima* 'Golden Delicious' with selected isolates of five fungal species (*F. culmorum*, *F. oxysporum*, *F. solani*,

Fig. 2. Box and whisker plots showing mean symptom ratings attributable to the inoculated fungus and the mock-inoculated control in greenhouse-grown *Cucurbita maxima* 'Golden Delicious'. **A**, Stem tissue, **B**, crown tissue, and **C**, root tissue. Symptoms were rated on a 1- to 4-scale that ranged from healthy (1) to severely diseased (4). Black dots indicate mean rating. Treatment labels: PC = *Plectosphaerella cucumerina*, S = *Setophoma terrestris*, FO = *Fusarium oxysporum*, FS = *F. solani*, and FC = *F. culmorum*.

P. cucumerina, and *S. terrestris*) resulted in symptoms of disease (foliar chlorosis, stem vascular discoloration, crown and root rot) from each of the fungi. Symptom expression began as early as 6 days after inoculation with *F. solani* and *F. culmorum*. Symptom ratings of specific host tissues (root, crown, and stem) recorded at the time of foliar symptom appearance or at the end of the experiment (49 days) showed that each species could cause symptoms of disease greater than the mock-inoculated control (Fig. 2, Table 4). Using MRPP, pairwise comparisons showed that *F. oxysporum*, *S. terrestris*, and *P. cucumerina* caused significantly higher crown and root rot symptoms compared with the control but did not cause significantly higher disease symptoms in the stem vascular tissue than control treated plants (Table 4, $P < 0.003$). *F. solani* and *F. culmorum* caused similarly severe disease symptoms in the stem and crown tissues (Table 4, $P < 0.003$). In root tissue, *F. culmorum* caused higher disease severity than *F. solani* ($P < 0.003$), and all fungal treatments had significantly greater disease severity than that of the mock-inoculated control (Fig. 2, Table 4).

For all five fungal species treatments, reisolation from the plants onto which they were inoculated was successful from at least 66% of stems, 81% of crown, and 87% of roots. *S. terrestris* was the least readily recultured species, despite the roots having characteristic pink rot symptoms, and *P. cucumerina* was more prevalent in the crowns and roots than stem tissue. When plated, tissue pieces from 45% of control plants yielded a few fungi that matched the morphology and molecular identity of the inoculated fungi; however, the isolation plates were dominated by other fungi, including many

Penicillium-type and *Mucorales*-type fungi. The identity of the reisolated pathogens was confirmed by sequencing the *ITS* region and achieving 98 to 100% identity.

C. maxima survey with unbiased sampling and crop rotation history in 2016. In 2016, a total of 4,497 fungal isolates were identified morphologically from a subset of 264 plants (of 1,632) and 263 isolates were identified by sequencing of the *ITS* and *EF1 α* genomic regions (Table 5). Fourteen fungal species were identified morphologically, and 27 species were identified from sequencing (Table 1). As in the 2014–15 survey, the most common fungal species recovered were *F. oxysporum* (25%), *P. cucumerina* (20%), *F. solani* (11%), *F. culmorum* (7%), and *S. terrestris* (4%) with other fungi accounting for the remainder (Supplementary Appendix S3; accession numbers MN521460 to MN521696). *P. cucumerina* and *S. terrestris* were recovered in greater frequencies from symptomatic than from healthy plants (Table 5). *Fusarium* spp., however, were cultured at the same or slightly lower rates in symptomatic than healthy plants. Compared with the other primary rotation crops, fields previously in grass seed had a greater incidence of *F. culmorum* (9% of isolates) and *F. solani* (13% of isolates) (Table 5). Plants within a processed vegetable primary rotation had the highest occurrence of *P. cucumerina* (26% of isolates). As in previous years, stem tissues yielded the fewest isolates and *P. cucumerina* was the most common species in this tissue (Table 5).

Overall, randomly collected plants were rated as healthy. For all 1,632 plants, the mean stem rating was $1.43 \pm (\text{SD}) 0.67$ (of possible 4), mean crown rating was 1.56 ± 0.72 , and mean root rating was

TABLE 4
Multiresponse permutation procedure (MRPP) analysis of symptom ratings of stems, crowns, and roots from inoculated *Cucurbita maxima* 'Golden Delicious' plants in greenhouse pathogenicity trials

Tissue	Inoculation treatment ^y	Significance group ^z	Inoculation treatment ^x					Control
			<i>Fusarium oxysporum</i>	<i>F. solani</i>	<i>Plectosphaerella cucumerina</i>	<i>F. culmorum</i>	<i>Setophoma terrestris</i>	
Stem	<i>F. oxysporum</i>	B	...	-10.826	-0.485	-13.178	-3.572	-4.823
	<i>F. solani</i>	A	<0.001	...	-17.296	-1.356	-21.998	-23.100
	<i>P. cucumerina</i>	B	0.206	<0.001	...	-19.238	-1.572	-3.573
	<i>F. culmorum</i>	A	<0.001	0.094	<0.001	...	-22.808	-23.633
	<i>S. terrestris</i>	B	0.012	<0.001	0.076	<0.001	...	Na
	Control	B	0.004	<0.001	0.011	<0.001	Na	...
Crown	<i>F. oxysporum</i>	B	...	-7.645	-0.268	-10.988	0.413	-12.471
	<i>F. solani</i>	A	<0.001	...	-9.870	-0.016	-12.353	-22.842
	<i>P. cucumerina</i>	B	0.291	<0.001	...	-14.610	-0.661	-19.002
	<i>F. culmorum</i>	A	<0.001	0.348	<0.001	...	-16.007	-23.522
	<i>S. terrestris</i>	B	0.548	<0.001	0.191	<0.001	...	-13.518
	Control	C	<0.001	<0.001	<0.001	<0.001	<0.001	...
Root	<i>F. oxysporum</i>	B	...	-1.700	0.010	-5.554	0.010	-11.144
	<i>F. solani</i>	AB	0.067	...	-2.957	-0.268	-2.957	-18.361
	<i>P. cucumerina</i>	B	0.338	0.017	...	-7.324	0.737	-15.297
	<i>F. culmorum</i>	A	<0.001	0.263	<0.001	...	-7.324	-22.988
	<i>S. terrestris</i>	B	0.338	0.017	0.829	<0.001	...	-15.297
	Control	C	<0.001	<0.001	<0.001	<0.001	<0.001	...

^x Values below the diagonal show *P* value of the likelihood that the distance between the two compared groups are based on chance alone. Bolded font indicates statistically significant comparisons adjusted for multiple comparisons using a Bonferroni correction and significant at the $P \leq 0.0033$ level. Values above the diagonal show *T* statistic calculated based on the within-group distances. More negative values show higher separation between compared groups.

^y Inoculated treatment groups used for pairwise comparisons. There were 18 plants treated in each inoculation treatment.

^z Significance groups as defined by pairwise MRPP *P* values. Treatments that are not significantly different from one another have the same letter.

1.67 ± 0.86 (Table 6). Plants from fields in the southern Willamette Valley (closer to processing centers) had numerically higher root symptom ratings than plants from northern fields, but northern fields had slightly higher stem and crown symptom ratings and percentage of dead plants. Plants from fields with a history of processed vegetables had comparatively higher root rot ratings (Figs. 3 and 4) and had the highest percentage of dead plants for all prior rotation crop types (Table 6). Similarly, plants from fields that had previously been planted in winter squash twice in the last 5 years had the highest mean crown and root ratings, 1.74 ± 0.72 and 2.10 ± 1.09, respectively, and the highest percentage of dead plants. Plants from fields with mint as the primary prior rotation crop had the lowest overall disease ratings for all tissue types.

MRPP supported the above observations with statistical evidence of group differences. Fields planted in the north valley had root symptom severities that differed significantly from fields planted in the south valley ($P \leq 0.05$) (Table 7). For each squash tissue, symptom severities from fields with a primary prior rotation of processed vegetables differed significantly from fields planted previously to mint ($P \leq 0.05$) (Table 7). Similarly, the comparison of root ratings for fields not in squash in the previous 5 years differed significantly from those planted to squash once or twice ($P \leq 0.05$).

Compared with crown and root symptoms, histograms of symptom rating distributions (Fig. 4) showed that stem tissue exhibited the least amount of variation among the environmental categories. In contrast, histograms for root symptom ratings showed the most variation with categories of “prior crop processed vegetable” or “planted to squash previously” having distributions skewed toward higher ratings relative to “other prior crops” or “not planted to squash previously.” χ^2 analysis of fungal species recovered from plants with high symptom ratings (3 or 4) compared with plants with low ratings (1 or 2) when grouped by prior primary rotation crop, also revealed differences in the frequency of recovery of some fungal species (Table 8). For the categories of prior crop processed vegetable or previously planted in squash, the frequency of recovery of *P. cucumerina* from stems and roots was significantly greater in plants with high symptom ratings compared with those with low ratings ($P \leq 0.05$) (Table 8). Similarly, frequency of recovery of *F. solani* increased in tissues with high symptom ratings compared with low symptom ratings for at least one tissue type in categories of primary prior crop processed vegetables, primary prior crop grass seed, previously planted in squash, and no squash previously ($P \leq 0.05$) (Table 8). In contrast, there were few differences among plants with high symptom ratings compared with

TABLE 5
Fungal species isolated from *Cucurbita maxima* plants collected from fields in Oregon’s Willamette Valley during the summer of 2016 summarized by method of isolate identification^u

Data set	Total count	Total percent	Number from diseased plants ^w	Number from healthy plants ^x	Number from primary prior crop ^v					Number from stem	Number from crown	Number from root
					Processed vegetables	Grass seed	Mint	Fallow	NA ^y			
Morphological												
<i>Plectosphaerella cucumerina</i>	867	19.28	657	210	266	339	39	1	222	389	302	178
<i>Fusarium oxysporum</i>	1135	25.24	799	336	204	517	116	1	297	154	545	436
<i>F. solani</i>	476	10.58	343	133	118	279	30	0	49	72	257	147
<i>F. culmorum</i>	324	7.20	222	102	47	190	33	0	54	143	107	74
Other <i>Fusarium</i> sp.	374	8.32	297	77	90	132	38	11	103	69	175	130
<i>Setophoma terrestris</i>	169	3.76	137	32	54	87	2	3	23	6	88	75
Other fungi	1,152	25.62	828	324	233	554	120	2	243	563	156	433
Total	4,497	100	3,283	1,214	1,012	2,098	378	18	991	1,396	1,630	1,473
Molecular ^z												
<i>P. cucumerina</i>	76	29	59	17	23	30	5	0	18	35	26	15
<i>F. oxysporum</i>	80	30	54	26	15	38	4	2	21	11	41	28
<i>F. solani</i>	2	1	2	0	2	0	0	0	0	0	1	1
<i>F. culmorum</i>	10	4	9	1	2	5	0	0	3	2	3	5
Other <i>Fusaria</i>	37	14	29	8	8	16	3	1	7	16	10	11
<i>Setophoma terrestris</i>	0	0	0	0	0	0	0	0	0	0	0	0
Other fungi	58	22	52	6	16	13	8	0	21	19	23	16
Total	263	100	205	58	68	102	20	3	70	83	104	76

^u Supplementary Appendix S1 provides information for all other fungi identified.

^v Primary prior crop was based on rotation histories provided by crop advisors or farmers.

^w Diseased plants received two or more ratings of 2 or at least one rating of 3 or 4. A total of 199 plants were identified as diseased.

^x Healthy plants received all 1 ratings or two 1 ratings plus one rating of 2. A total of 65 plants were identified as healthy.

^y Rotation history unknown.

^z Isolates identified through sequencing of *ITS* and *EF1 α* genes.

those with low ratings for *F. oxysporum*, *F. culmorum*, and *S. terrestris*.

DISCUSSION

A specific community of fungi was found to be associated with winter squash distributed among stem, crown, and root tissues and made up of *F. oxysporum*, *F. solani*, *F. culmorum*, *P. cucumerina*, and *S. terrestris*. These species have been identified as pathogens of cucurbits in previous studies (Chilosi et al. 2008; García-Jiménez et al. 2008; Gwynne et al. 1997; Hawthorne 1988; Ikeda et al. 2012). However, other reported cucurbit pathogens such as *Acremonium* spp., *Monosporascus* spp., *Pythium* spp., or *Rhizopycnis* spp., which have been associated with melon and cucurbit vine collapse diseases, were not detected (Aegerter et al. 2000; Al-Sadi et al. 2011; Armengol et al. 2003; Chilosi et al. 2008; Martyn and Miller 1996). This study demonstrated that these fungi were not exclusive to *C. maxima*-type cucurbits but occurred in all cucurbit species grown for commercial production in the Willamette Valley. Differences in fungal communities associated with winter squash grown in grass seed, processed vegetable, mint, fresh market, or fallow rotations were also observed.

The survey data presented here suggests that some fungal species consistently detected in squash tissues could be more important than others with respect to crop health and productivity. *P. cucumerina* was the only pathogen found consistently associated with stem tissue, and in 2016, was commonly cultured from plants with high symptom ratings. This fungus was also associated with fresh market and processed vegetable fields. However, it was not highly virulent in pathogenicity trials and did not readily replicate vascular discoloration symptoms, possibly due to the pathogenicity test being conducted only on seedlings, while survey disease ratings and cultures were collected from mature plants (Gerlagh and Blok 1988). This fungus has been studied in cucurbit collapses in Italy and Spain and there is potentially pathogenic specialization within *Plectosphaerella*, which at this time is not well understood

(Carlucci et al. 2012; Chilosi et al. 2008). *P. cucumerina* has a wide host range, having been cultured from bamboo, cabbage, endive, poplar, potatoes, soybean, sunflower, tomatoes, and other crops (Arzanlou et al. 2013; Carlucci et al. 2012; Gao et al. 2016; Garibaldi et al. 2013; Li et al. 2017; Roy 1997; Xu et al. 2014; Zhang et al. 2015), which may be why it was isolated more frequently from plants sampled from fresh market vegetable farms that have highly varied rotations. Although the field sampling and pathogenicity tests appear to be at odds, *P. cucumerina* is a complex species exhibiting a wide range of host–fungus interactions, including generating root rot, foliar blights, and leaf spots, and even occurring as an endophyte (Arzanlou et al. 2013; Carlucci et al. 2012; Gao et al. 2016; Garibaldi et al. 2013; Li et al. 2017; Su et al. 2017). It is possible that *P. cucumerina* may intensify disease symptoms when in combination with another fungus.

F. oxysporum was the species isolated most frequently from both healthy and symptomatic squash plants; however, in greenhouse pathogenicity trials, the inoculated isolates were only mildly virulent. These results may reflect that both pathogenic and non-pathogenic strains of *F. oxysporum* can be associated with roots of cucurbits (Gordon et al. 1989; Stergiopoulos and Gordon 2014). Pathogenic specialization in *F. oxysporum* (i.e., *formae speciales*) has been identified for other cucurbits but it has not been identified frequently in *C. maxima*-type squash (Edel-Hermann and Lecomte 2019). Given the high incidence of *F. oxysporum* in surface-disinfested squash tissues, the survey results suggest it is possible that both pathogenic and nonpathogenic strains were isolated, but that the fungus was not a severe root rot pathogen without the presence of other pathogens (Gordon et al. 1989; Gwynne et al. 1997; Zuniga et al. 1997).

In the 2016 survey of randomly collected plants, *F. solani* was frequently associated with crowns and roots of plants with high symptom ratings and was highly virulent in the pathogenicity trials, however, it was recovered less commonly than *F. oxysporum*. As early as 1938 in California, *F. solani* f. sp. *cucurbitae* was identified as a root, crown, and fruit rot pathogen of *C. pepo* cultivars (Gries

TABLE 6
Summary of symptom rating (1 to 4 scale)^v in stem, crown, and root tissues of *Cucurbita maxima* ‘Golden Delicious’ plants collected from 49 fields in Oregon’s Willamette Valley during the 2016 growing season

Comparison	Group (number of plants)	Mean stem rating (SD)	Mean crown rating (SD)	Mean root rating (SD)	% Plants dead at collection ^w
Field location ^x	South fields (1,196)	1.41 (0.65)	1.54 (0.72)	1.70 (0.87)	5 a
	North fields (436)	1.47 (0.70)	1.63 (0.72)	1.56 (0.82)	7 a
Primary prior crop ^y	Vegetable (366)	1.47 (0.68)	1.69 (0.75)	1.77 (0.94)	10 a
	Grass seed (693)	1.42 (0.68)	1.52 (0.72)	1.68 (0.83)	7 ab
	Mint (159)	1.31 (0.50)	1.44 (0.62)	1.40 (0.73)	3 ab
	Fallow (20)	1.80 (0.95)	1.90 (0.91)	1.70 (0.80)	0 b
Years previously in squash production ^z	0 Years in squash (932)	1.40 (0.65)	1.53 (0.70)	1.57 (0.80)	6 a
	1 Year in squash (247)	1.55 (0.75)	1.66 (0.80)	1.96 (0.92)	10 a
	2 Years in squash (59)	1.38 (0.53)	1.74 (0.72)	2.10 (1.09)	15 a
All plants	All plants (1,632)	1.43 (0.67)	1.56 (0.72)	1.67 (0.86)	6

^v Rating scale for stem, crown, and root tissue: 1 indicates no yellowing/discoloration, 2 indicates localized yellow to orange discoloration, 3 indicates localized orange to brown discoloration, and 4 indicates complete discoloration.

^w Percentages with the same letters are not significantly different in a test of equal proportions for group comparisons. Percentages with different letters are significantly different ($P \leq 0.05$) in a test of equal proportions for group comparisons.

^x Field location in the Willamette Valley as defined by its relation to the 45° latitude line.

^y Primary prior crop as determined by 5-year rotation history provided by growers and field representatives.

^z Number of times that the field had been planted in winter squash during the last 5 years according to representatives.

1946; Snyder 1938). There are two distinct pathogen races of *F. solani* f. sp. *cucurbitae* that have been identified: race 1 infects roots, stems and fruit, and race 2 only infects fruit (Elmer et al. 2007; Samac and Leong 1989). The ability of this fungus to cause disease in *C. maxima* cultivars has been investigated in the United

States and Japan, where after root inoculation, *C. maxima* cultivars were observed to be more susceptible than cultivars of *C. moschata*, *C. pepo*, and *Cucumis sativus* (Nagao et al. 1994; Samac and Leong 1989). Nonetheless, while *C. maxima* is one of the more susceptible cucurbit hosts of *F. solani*, most recent investigations have focused

Fig. 3. Histograms of symptom ratings of stems, crowns, and roots of *Cucurbita maxima* 'Golden Delicious' plants sampled from fields in the Willamette Valley of Oregon in 2016 categorized by grouping factors of **A**, primary prior crop and **B**, number of years of previous 5 years the field was planted to squash. Symptoms were rated on a 1- to 4-scale that ranged from healthy (1) to severely diseased (4). Numbers above each bar are counts of the number of plants in each rating group.

on *C. pepo* (Mehl and Epstein 2007). Because *F. solani* can be infective on other cucurbit hosts, this may explain its stronger association with fresh market fields, particularly those that produce multiple species of cucurbits on shorter rotations. *F. solani* has the potential to be an economically relevant pathogen in winter squash production in Oregon, but it was not as commonly recovered from

surveyed symptomatic plants, suggesting the need for another pathogen to be present for adequate virulence (Mehl and Epstein 2007; Zhang et al. 2006).

F. culmorum was not as commonly cultured as the other *Fusarium* species but was associated with grass seed fields, which is a very common rotation crop in the Willamette Valley. Grass

Fig. 4. Mean symptom ratings for **A**, stem, **B**, crown, and **C**, root tissues on a 1 (healthy) to 4 (diseased) disease severity scale from plants of *Cucurbita maxima* 'Golden Delicious' collected in 2016 compared with the number of years the field was planted in squash in the last 5 years. Shapes represent primary prior crop and colors represent the location (north or south of the 45° latitude line) in the Willamette Valley of Oregon.

species are common hosts of *F. culmorum*, where it causes important diseases such as Fusarium head blight (Leslie and Summerell 2006). *F. culmorum* has been identified as a causal agent of postharvest fruit rot of winter squash (Hawthorne 1988; Rivedal et al. 2018a). *F. culmorum* was highly virulent in the greenhouse pathogenicity trials conducted in this study on seedlings. The higher incidence of this organism in fields cropped previously to grass seed may be why some squash fields had relatively higher symptom ratings despite no recent history of squash or other vegetables. The data from mature plant surveys and greenhouse bioassays were conflicting, likely due to the fact that the disease in the field is a mature plant disease and not a seedling disease, which changes cucurbit susceptibility (Gerlagh and Blok 1988).

S. terrestris was associated with a pink root rot symptom on squash, which was replicated in the greenhouse pathogenicity experiment. Compared with severe symptoms caused by *F. solani*

and *F. culmorum*, symptoms caused by *S. terrestris* were relatively minor and the pathogen did not kill any of the inoculated plants. This pathogen causes pink root in other vegetable crops, notably *Allium* spp., and has been described as a pathogen of cucurbits in Japan and the United States (De Gruyter et al. 2010; Ikeda et al. 2012; Rivedal et al. 2018b). The survey data and pathogenicity trial suggest that *S. terrestris* is probably not a major pathogen of *C. maxima* on its own. The survey results, however, leave open the question that the five most common fungi may take on greater significance when they co-occur in a host.

Unsurprisingly, previously planted crop was at least a partial predictor of symptom severity, and like others (Rowe and Powelson 2002; Stergiopoulos and Gordon 2014), the present study found that crop rotation patterns contributed to an increase or decrease in certain fungal species. Specifically, those fields that had been planted in fresh market or processing vegetable rotations had an increase in occurrence of *F. solani* and *P. cucumerina*, while grass

TABLE 7
Multiresponse permutation procedure (MRPP) analysis of symptom ratings of stems, crowns, and roots from survey of *Cucurbita maxima* 'Golden Delicious' plants collected from fields in Oregon's Willamette Valley in 2016

Groups compared ^w		Number of plants per group ^x	Stem		Crown		Root	
			<i>t</i> statistic ^y	<i>P</i> value ^z	<i>t</i> statistic ^y	<i>P</i> value ^z	<i>t</i> statistic ^y	<i>P</i> value ^z
Valley location <i>P</i> ≤ 0.05	South vs. north	856 vs. 292	-0.29	0.24	-3.08	0.02	-5.11	<0.01
Primary prior crop <i>P</i> ≤ 0.0083	Vegetables vs. grass seed	331 vs. 643	-0.71	0.16	-9.88	<0.01	-1.49	0.08
	Vegetables vs. mint	331 vs. 154	-2.65	0.03	-8.16	<0.01	-12.15	<0.01
	Vegetables vs. fallow	331 vs. 20	-0.68	0.17	0.33	0.49	0.30	0.47
	Grass seed vs. mint	643 vs. 154	-0.80	0.15	0.01	0.33	-12.56	<0.01
	Grass seed vs. fallow	643 vs. 20	-1.76	0.06	-2.06	0.05	0.69	0.76
	Mint vs. fallow	154 vs. 20	-3.30	0.02	-3.00	0.02	-2.44	0.03
Years previously planted in squash <i>P</i> ≤ 0.016	0 Years vs. 1 year	875 vs. 223	-3.91	0.01	-1.73	0.06	-25.76	<0.01
	0 Years vs. 2 years	875 vs. 50	-0.09	0.30	-3.77	0.01	-7.82	<0.01
	1 Year vs. 2 years	223 vs. 50	0.47	0.56	-1.04	0.12	0.04	0.36

^w Groups compared in MRPP analysis, same as those defined for nonmetric multidimensional scaling comparisons.

^x Number of plants being compared in each specified group with MRPP.

^y *t* statistic calculated based on the within-group distances. More negative values show higher separation between compared groups.

^z *P* value from a Monte Carlo test of significance for observed indicator value compared with that expected at random. Bolded values indicate statistically significant *P* values of ≤0.05.

TABLE 8
P values from χ^2 tests of independence for selected fungal species isolated in 2016 from *Cucurbita maxima* 'Golden Delicious' plants^u with low symptom ratings compared with plants with high symptom ratings^v categorized by primary prior crop and by if the fields were planted to squash in the last 5 years^w

Isolate	Grass seed				Vegetables				No squash previously				Previously planted in squash ^x			
	Stem ^y	Crown ^y	Root ^y	Total ^z	Stem	Crown	Root	Total	Stem	Crown	Root	Total	Stem	Crown	Root	Total
<i>Plectosphaerella cucumerina</i>	0.61	0.46	0.06	0.11	<0.01	0.75	<0.01	<0.01	0.39	0.87	0.18	0.28	<0.01	0.73	<0.01	<0.01
<i>Fusarium oxysporum</i>	0.44	0.30	0.45	0.32	0.02	0.37	0.459	0.03	0.90	0.93	0.87	0.97	0.04	0.16	<0.01	<0.01
<i>F. solani</i>	0.24	0.04	<0.01	<0.01	0.92	<0.01	0.01	<0.01	0.09	0.22	<0.01	<0.01	<0.01	0.10	0.91	<0.01
<i>F. culmorum</i>	0.95	0.47	0.47	0.59	0.90	0.27	0.20	0.24	0.78	0.41	0.49	0.54	0.98	0.25	0.16	0.19
<i>Setophoma terrestris</i>	0.78	0.42	0.37	0.46	0.38	0.55	0.75	0.54	0.59	0.17	0.16	0.13	0.25	0.02	0.04	<0.01

^u All collections made from commercial squash fields in the Willamette Valley, OR.

^v Low symptom ratings are 1 or 2, and high symptom ratings are 3 or 4.

^w Bolded values indicate statistically significant *P* values of <0.05.

^x One or two years of the 5 previous years planted in winter squash.

^y χ^2 test performed between only stem, crown, or root percentages.

^z χ^2 test performed with all three percentages for the fungal isolate.

seed as a prior crop had an increase in occurrence of *F. culmorum*. For both primary prior rotation crop and years previously planted in squash, the 2016 survey results showed that incidence of *F. solani* and *P. cucumerina* in the fungal community increased in plants with high symptom ratings compared with plants with low ratings. These fungal species may be necessary members of the community for disease development in winter squash.

Because this study was unable to show a large difference between the fungal communities of symptomatic and healthy plants, it is possible that unknown environmental and/or edaphic factors also contribute to symptom expression. For example, in Indiana, Makam et al. (2005) found that cultural conditions such as planting into plastic mulch, low pH, low organic matter, and preemergent herbicide treatments resulted in lower root fresh weight and vine decline symptoms when no causal agent could be identified in watermelon. Additionally, atmospheric and soil temperature are known to enhance soilborne melon collapse caused by *Monosporascus cannonballus* and *Acremonium cucurbitacearum* in Texas and Israel (Bruton et al. 1999; Pivonia et al. 2002). Temperature at planting and during stressful periods within the growing season, such as fruit set, may also contribute to incidence and severity of winter squash decline (Bruton et al. 1999). Another potential driver of root rot disease severity in the Willamette Valley could relate to water use (Al-Mawaali et al. 2013; Pivonia et al. 1997). Most winter squash farmers apply overhead irrigation water throughout the season. In potato, Powelson and Rowe (1993) showed that potato early dying symptom severity late in the season was enhanced by excessive soil moisture early in the season, and that both symptoms and yield losses could be reduced by imposing mild drought stress conditions in the period before tuber initiation. Current investigations are being conducted on whether mild drought stress prior to fruit set in winter squash can reduce root rot disease severity.

This study relied on culturable fungi as a basis for the short list of potential pathogens causing winter squash decline because of the similarity in symptoms to previously mentioned cucurbit declines. Due to these methods, it is possible that a causal agent was not captured, such as a bacterial, obligate, or oomycete pathogen. However, in intensive pathogenicity trials that incorporated mixed inoculum sources, symptoms like those seen in the field were recreated, suggesting that this is a problem caused by the fungi collected in these surveys (H. M. Rivedal, A. G. Stone, J. F. Tabima, and K. B. Johnson, unpublished data).

Until recently, winter squash has not been a common rotation crop for grass seed or processed vegetable producers of the Willamette Valley. For example, in this effort, most (78%) surveyed fields with rotation histories had less than 1 year of squash production in the last 5 years. In spite of this, unhealthy plants were found in every surveyed field, and rotation crops did affect the prevalence of certain fungi associated with winter squash. Consequently, this study clearly indicates squash yield decline is a complex problem and that crop performance is difficult to predict due to the involvement of several potential pathogens and the potential contribution of several environmental factors. Importantly, this effort provides a framework on which to base future investigations into causal agent pathogens and management strategies to ameliorate this problem.

ACKNOWLEDGMENTS

We thank the crop advisors from Stahlbush Island Farms and Autumn Seed for providing farmer contacts and field recommendations; winter squash growers for access to their land and rotation history records; greenhouse operations staff at the OSU West Greenhouses for their technical support of the pathogenicity trials; members of the Johnson and Stone labs, specifically Todd N. Temple,

Evan C. Thompson, Victoria V. Smith, Brandon L. Ferington, Mora A. Camplair, and Gabriella M. Boyer, for their technical assistance with inoculations and data collection; and Duncan R. Kroese for technical assistance with data collection and general support.

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