New Outbreaks of Verticillium Wilt on Hop in Oregon Caused by Nonlethal Verticillium albo-atrum

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Abstract
In 2006 and 2007, outbreaks of Verticillium wilt on hop were detected on two farms in Oregon. Some Verticillium pathogens cause minor damage to hop but others can kill susceptible cultivars: strains of lethal V. albo-atrum are not known to occur in Oregon. Studies were conducted to determine the identity of the Verticillium species associated with these outbreaks and their virulence to hop to devise an appropriate response to the outbreaks. Isolates were recovered from wilted plants, and identified as V. albo-atrum based on morphological characters and ITS region sequence. Pathogenicity assays conducted on a set of differential cultivars indicated all isolates had a low to intermediate level of virulence. Inspection and mapping of all symptomatic and dead plants in a hop yard on an affected farm indicated that 29.3% and 19.7% of plants had symptoms of Verticillium wilt in 2006 and 2007, respectively. However, the number of dead or missing plants did not increase from 2006 to 2007 (0.26% in both years). There was a clear directionality to the pattern of symptomatic plants among rows running north-south vs. east-west in 2006, although directionality was less pronounced in 2007. These findings indicate that the recent outbreaks of V. albo-atrum on hop in Oregon were caused by nonlethal strains, which has key implications for devising management strategies on the affected farms.

Introduction
Vascular wilts caused by Verticillium albo-atrum and V. dahliae are important diseases on a broad range of mostly dicotyledonous host plants throughout the world (14). Management of these diseases in perennial crops can be particularly difficult due to the impracticality of crop rotation and fumigation after crop establishment. Consequently, management often relies on modified cultural practices and resistant cultivars, when available (14).

Both V. albo-atrum and V. dahliae can cause a wilt disease on hop (Humulus lupulus), with V. albo-atrum being the primary wilt pathogen in most hop production areas worldwide (13). V. albo-atrum is known to occur very infrequently on hop in Oregon (1) and is thought to be localized to a limited number of yards on only a few hop farms. The pathogen has not been reported from hop in Washington State. Strains of V. albo-atrum vary in their virulence to hop, and have been categorized as "fluctuating" (mild) or "progressive" (lethal) strains based on characteristics of the disease outbreaks caused by these strains and their virulence on differential cultivars (8,9,15,21). Sewell and Wilson (20) refer to four virulence groups, "mild" (M) and "progressive" (PV) strains PV1, PV2, and PV3, which display increasing virulence from M to PV3 that can be moderated by environmental conditions. They suggested that variations in virulence appear to be "more or less continuous" but note the convenience and practical value of categorical strain designations for delineating relative pathogenicity (18).
Radišek (15) described mild Verticillium wilt caused by \textit{V. albo-atrum} resulting from the occurrence of a less virulent strain of the pathogen in a susceptible cultivar or alternatively a virulent strain on a resistant cultivar. Mild Verticillium wilt tends to vary in severity from year to year in an infested hop yard in response to soil temperature, nitrogen fertilization, and other environmental factors (20). Plants affected by the mild form of the disease in one year generally recover and appear healthy during the ensuing year. At the field level the rate of increase in disease incidence appears slow (9). In contrast, lethal Verticillium wilt results in wilt of whole plants and death in susceptible cultivars. Occurrence of disease is less influenced by weather conditions and soil factors. Disease incidence tends to spread and increase rapidly in affected yards. With lethal Verticillium wilt, the pattern of infected plants tends to be related to the primary direction of soil cultivation operations, and disease incidence tends to increase in successive years after introduction of the pathogen (9). Outbreaks of lethal Verticillium wilt have been reported from England, Germany, and Slovenia (15).

In contrast, \textit{V. dahliae} generally is regarded as a weak and only occasional pathogen on hop, mostly in warmer climates, and infected plants may not exhibit obvious wilt symptoms (14,15). This pathogen was first isolated from the Verticillium wilt susceptible cultivar Fuggle in Oregon in 1956 (7). Later, Zehsazian (22) concluded that isolates of \textit{V. dahliae} from hop in Oregon were "not virulent pathogens of this crop" and were adapted primarily to other hosts, as later demonstrated for \textit{V. dahliae} on other hosts (3). On the cultivar Willamette, a triploid derived from cultivar Fuggle (4), \textit{V. dahliae} was isolated from hop plants in 13 of 20 yards surveyed in western Oregon (1). Infected plants generally displayed a swelling of the bine and chlorosis and marginal necrosis of older leaves without appreciable wilting of bines or associated yield loss. In this thesis, Barth (1) concluded that \textit{V. dahliae} does not severely affect development of Willamette or other hop cultivars susceptible to \textit{V. albo-atrum}.

In 2006, a new outbreak of Verticillium wilt was detected on a hop farm in western Oregon, affecting several cultivars including Willamette and Nugget, the two most widely planted cultivars in Oregon. The grower noted, anecdotally, that wilted bines were observed since at least 2001 and that the incidence of affected plants had increased since then. In 2007, wilted hop plants were observed on a second farm in a newly established hop yard planted to cultivar Willamette. This second field had not been planted to hops since 1991, although Barth (1) had previously recovered \textit{V. albo-atrum} from hop plants with Verticillium wilt in this field. \textit{Verticillium} pathogens are readily disseminated in planting materials (9), and since hop rootstock is distributed internationally the outbreaks of Verticillium wilt prompted immediate concern that a lethal strain of \textit{V. albo-atrum} had been introduced. Such an introduction could have serious implications for the United States hop industry since over 99% of the production occurs in the United States Pacific Northwest and nearly all hop cultivars currently grown in the USA are susceptible to lethal strains of \textit{V. albo-atrum} (6). Information on the identity and virulence of the wilt pathogen(s) is critical to deploy appropriate management tactics. Due to the potential for catastrophic crop losses that may result from lethal Verticillium wilt, these new outbreaks prompted us to conduct studies to: (i) identify the \textit{Verticillium} pathogen(s) associated with the disease by morphological characters and molecular methods; (ii) characterize the virulence of isolates; and (iii) describe the spatial pattern of affected plants to infer if the outbreaks were caused by a mild or lethal form of the disease.

**Isolate Collection and Virulence Assays**

During 2006 and 2007, a total of 40 stems from wilted bines were collected from two hop yards (cultivar Nugget and Willamette and referred to as farms ‘A’ and ‘B,’ respectively) in western Oregon. The affected plants exhibited swelling of the lower portion of the bines, interveinal chlorosis and necrosis, upward curling of leaves, and wilting of some entire bines (Fig. 1). Sections of stems were collected from the bases of symptomatic plants selected arbitrarily in each yard. The bark was peeled from the stems and thin slices were cut from the stem...
and sectioned into pieces approximately 1 mm square. These pieces were surface disinfested in 1% bleach for 60 sec, rinsed in sterile 18 ohm water, blotted dry, and placed on water agar. A Verticillium species was isolated from each affected stem, yielding 40 isolates in total. Transfers were made to potato dextrose agar (PDA) amended with 100 µg/ml streptomycin, identified as a Verticillium spp. by morphological characters, and then single spore cultured. In total, 16 single spore isolates were collected.

Isolates were identified to the species level based on the presence/absence of microsclerotia after 14 to 21 days of growth on PDA at 18°C in the dark. Production of resting structures on artificial growth media is frequently used as a means to distinguish V. albo-atrum and V. dahliae, although resting structures may not be produced after prolonged in vitro culture (2). V. albo-atrum produces only dark-walled resting mycelia whereas V. dahliae produces microsclerotia. All 40 Verticillium spp. recovered were identified as V. albo-atrum based on these characteristics. Four single spore isolates from yard A were selected for sequencing of the ITS region as previously described (11). These regions (ITS1, 5.8S rDNA, and ITS 2) were consistent with V. albo-atrum (100% sequence similarity to the 452 nucleotide sequence of GenBank accession AF364015). Isolates were maintained on PDA at 4°C in the dark until needed for a virulence assay as described below.

Greenhouse bioassays were conducted to assess virulence of six isolates on the Verticillium wilt differential cultivars Fuggle, Whitbread’s Golding Variety (WGV), and Wye Target (18). Isolates of the pathogen with low virulence on
Fuggle are classified as M, isolates with low virulence on WGV and Wye Target are classified as PV1, isolates causing severe wilting on WGV but not Wye Target are classified as PV2, and isolates causing severe symptoms on Wye Target are classified as PV3 (18).

Plants were propagated from soft wood cuttings, rooted in perlite, and maintained in a greenhouse at approximately 20 to 25°C with at least a 14-h photoperiod. Plants were grown in Sunshine Mix #1 (SunGro Horticulture, Bellevue, WA) in 1000 cm³ pots, watered regularly, and fertilized using Champion 17-17-17 (N-P₂O₅-K₂O) with micronutrients (McConkey Co., Sumner, WA) at each irrigation. Five to 10 plants of each cultivar were selected and inoculated with each isolate individually by means of a root clip method. Plants were removed from the perlite and the roots were carefully rinsed in tap water. The roots were clipped to approximately half of their original length with sterile scissors and plants were then placed in a suspension of 10⁶ conidia/ml. The plant was exposed to the inoculum for 30 min with active stirring by a magnetic stir bar, and then repotted. Control plants were treated similarly but placed in sterile deionized water. After inoculation, plants were maintained as a single primary bine and wilt symptoms were assessed as described by Radišek et al. (17). Beginning 4 weeks after inoculation, the severity of Verticillium wilt was assessed visually using a 0-to-5 scale based on the proportion of symptomatic foliage, where 0 = no leaf symptoms, 1 = 1 to 20% leaf area wilted, 2 = 21 to 40% leaf area wilted, 3 = 41 to 60% leaf area wilted, 4 = 61 to 80% leaf area wilted, and 5 = 81 to 100% leaf area wilted or a dead plant. Ratings were repeated biweekly for an additional four weeks. The assays were repeated once using new plants.

The six *V. albo-atrum* isolates utilized in the assays varied in their virulence on the differential cultivars, although none of the isolates appeared highly virulent to Wye Target (Table 1). Five of the six isolates were virulent on Fuggle (mean rating of 3 or higher), and one of the six isolates was virulent on WGV. Due to the low virulence of isolate 58 on Fuggle, which was consistent in repeated inoculations, this isolate would be classified as mild (‘M’). Isolates 41, 56, 58, and 59 are classified as PV1 based on their virulence to Fuggle but low virulence to WGV and Wye Target. Isolates 55 and 57 were virulent to Fuggle, but induced more severe wilt symptoms on WGV as compared to the PV1 isolates. This pattern of disease indicates a virulence that is intermediate to PV1 and PV2 isolates.

<table>
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<tr>
<th>Isolate</th>
<th>Mean disease rating (std. dev.)*</th>
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<tr>
<td></td>
<td>Fuggle</td>
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<tr>
<td>Water</td>
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<tr>
<td>41</td>
<td>4.5</td>
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<td>55</td>
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<tr>
<td>58</td>
<td>1.3</td>
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<tr>
<td>59</td>
<td>3.0</td>
</tr>
<tr>
<td>All isolates</td>
<td>3.5 (1.04)</td>
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*Plants were inoculated using a root clip method. Verticillium wilt severity was assessed using a 0-to-5 scale based on the proportion of symptomatic foliage, where: 0 = no leaf symptoms; 1 = 1 to 20% leaf area wilted; 2 = 21 to 40% leaf area wilted; 3 = 41 to 60% leaf area wilted; 4 = 61 to 80% leaf area wilted; and 5 = 81 to 100% leaf area wilted or a dead plant. Data are means from two runs of the experiments with standard deviation of the disease ratings from all isolates presented parenthetically.*
Spatial Analysis of Disease Patterns

As described previously, the pattern of affected plants can provide some indication of whether a hop yard is affected by a mild or lethal form of Verticillium wilt based on the rapidity of disease spread and seasonal fluctuations in disease severity. Hop plants in a yard planted to cultivar Nugget on farm ‘A’ were assessed for symptoms of Verticillium wilt in both 2006 and 2007. Hills of plants (groups of one or more hops planted together) were arranged on a narrow row configuration where hills are evenly spaced in a 2.3-m × 2.3-m lattice pattern. Disease assessments were conducted on 8 and 9 August during 2006, and 13 and 14 August during 2007. All of the hop plants in a rectangular section of the yard measuring 70 rows (2006) or 69 rows (2007) wide by 163 hills long were inspected and the coordinates of diseased or missing (dead) plants was recorded. A hill was considered dead if no green shoots were present at the time of the disease ratings. There were a total of 11,130 and 10,971 hills (including hills with missing plants) assessed in 2006 and 2007, respectively. Hop plants with at least one wilted bine were considered diseased. To verify that plants recorded as diseased were actually affected by Verticillium wilt, periodically symptomatic plants were arbitrarily selected and stems were cut longitudinally and inspected for vascular browning. All stems (from more than 100 plants) inspected had characteristic swelling and vascular browning that is diagnostic for Verticillium wilt, and V. albo-atrum was recovered from all stems collected from this yard as described above.

Runs analyses were performed to characterize the pattern of diseased and missing plants in both the north-south direction and east-west direction. Each hill was assigned a value of "1" if the hill was rated as diseased or "0" if it was rated as non-diseased. A run is defined as succession of one or more plants with similar disease status (non-diseased or diseased) (10). For example, a sequence of "0-0-1-1-0-1-1" contains four runs (0-0, 1-1-1, 0, and 1-1). The expected number of runs was calculated and used to produce a Z-statistic to test the null hypothesis that the number of runs was not different significantly ($P \leq 0.05$) from the expected number of runs, i.e., an indication of a random distribution of disease among plants. Runs were calculated in Minitab version 15 (Minitab Inc., State College, PA).

In both 2006 and 2007, wilting of bines associated with Verticillium wilt was widespread in yard ‘A,’ although disease incidence was less in 2007 compared to 2006 (Fig. 2). Of the 11,130 hills assessed in 2006, a total of 2,699 hills (29.3%) had at least one bine that was completely wilted and 29 hills (0.26%) were rated as dead. Of the 10,971 hills assessed in 2007, 2,162 hills (19.7%) had a least one wilted bine and 28 hills (0.26%) were dead. Generally, only one or two bines were wilted on affected hills. In neither year were hills observed where all of the bines were wilted.

There was a greater degree of aggregation of affected plants in rows running north-south compared to east-west, particularly in 2006. In 2006, runs analysis indicated significant aggregation in 40 of the 70 rows (57.1%) running north-south, but only 10 of 159 rows (6.3%) in the east-west direction. In contrast in 2007, significant aggregation was detected in 14 of the 69 rows (20.3%) running north-south, and 26 of 159 rows (16.4%) in the east-west direction.
Fig. 2. Spatial pattern of hop plants (cultivar Nugget) affected by Verticillium wilt caused by *Verticillium albo-atrum* in 2006 (left) and 2007 (right). Small open circles indicate hills (groups of one or more plants) with at least one wilted bine. The larger closed circles indicate hills where no green shoots were observed at the time of the disease ratings (8 and 9 August 2006, and 13 and 14 August 2007) and were presumed dead. Note that spring pruning of the hills was conducted by mechanical means in the north-south direction in this yard. Hop debris remaining after harvest was spread on the south end of the hop yard in 2005 but not in 2006.
**Discussion and Recommendations**

Deployment of appropriate management strategies for Verticillium wilt on hop requires information on the identity and virulence of the pathogen(s) involved with the disease. In this study, outbreaks of Verticillium wilt observed on two farms in western Oregon were caused by *V. albo-atrum*. *V. dahliae* was not recovered from the two hop yards sampled in this study. The outbreak of Verticillium wilt in cultivar Nugget on one of the farms is the first confirmed instance of *V. albo-atrum* on this farm, which suggests that the pathogen was recently introduced or has spread among hop yards in Oregon. The re-emergence of *V. albo-atrum* on a farm where Verticillium wilt had not observed for 19 years is also significant, and suggests a potential for extended survival on non-hosts and possible resurgence (19).

Importantly, the virulence of the isolates associated with these outbreaks would be classified as M, PV1, or, for two isolates, perhaps PV2 based on the classification system utilized by Sewell and Wilson (18). To our knowledge, this is the first classification of *V. albo-atrum* strains from hop in Oregon using this classification system. Previous studies have utilized stem inoculations (16,17) or soil infestation [e.g., (18)], ideally with incubation under natural conditions (8,18). Resistance to stem and root colonization by *V. albo-atrum* may vary in hop, and the intrusive inoculation procedure used in this study may potentially overestimate the virulence of the isolates. Moderation of environmental conditions on symptom expression was not controlled for in the current study since inoculations were conducted under greenhouse conditions. Ideally, these virulence assays should be conducted under natural conditions over multiple seasons to account for seasonal differences in potentially moderating environmental conditions. Such an approach was not possible (nor advisable) in the current study given the very limited distribution of *V. albo-atrum* on hop in Oregon and lack of plots where *V. albo-atrum* could be introduced.

The spatial patterns of plants affected by Verticillium wilt in hop yard A in 2006 and 2007 support the suggestion that the strains of *V. albo-atrum* present do not appear to be highly virulent. Disease incidence assessed in early to mid August decreased 9.6% in 2007 compared to 2006, and certain plants with wilted bines in 2006 appeared healthy in 2007. Importantly, the number of plants rated as dead did not increase following 2006. It is also relevant to note that the cultivar Nugget (5) is regarded as susceptible to Verticillium wilt in Europe (12). That extensive plant death was not observed in the field is further suggestive of a nonlethal strain of *V. albo-atrum*. Therefore, the attributes of the outbreaks are characteristic of non-lethal strains of *V. albo-atrum* (15) and support the virulence ratings determined by the greenhouse bioassays.

Under controlled conditions, the severity of Verticillium wilt caused by non-lethal strains of *V. albo-atrum* increased on average 9% for every 1°C decrease in soil temperature (19). The greater incidence of plants with Verticillium wilt symptoms in 2006 (29.3%) versus 2007 (19.7%) could be due in part to warmer soils during late winter and spring. Weather data for 2006 and 2007 were obtained from the Aurora AgriMet weather station maintained by the United States Bureau of Reclamation situated approximately 12 km from the field indicated that mean daily soil temperature at a depth of 20 cm were 11.0, 15.8, 20.5, and 23.5°C during March, April, May, and June 2006, respectively, and 13.5, 14.2, 17.0, and 21.2°C during the same months in 2007. Rainfall during these months was 28.2 cm in 2006 and 20.0 cm in 2007.

It is interesting to note that the pattern of diseased plants was more strongly aggregated in the north-south direction of the hop yard in 2006 as compared to 2007. It is uncertain why such a difference in aggregation was observed. Mechanical pruning of hills in spring was conducted in both years in the north-south direction. Later cultivation for weed control was in both the east-west and north-south direction early in the season (up late June), but only north-south later in the season. The grower did report that crop debris remaining after harvest was not spread on this yard following harvest in 2006, as had been done in the past, although it is not clear if this management change alone would affect the pattern of disease aggregation so prominently.
Rational and effective management options for a disease outbreak cannot be devised until the nature of a variable pathogen has been determined. Significantly, our overall findings are that recent outbreaks of Verticillium wilt in Oregon were caused by a nonlethal strain of *V. albo-atrum*. It is important to note that lethal strains of *V. albo-atrum* are still not known to occur in this region (1,22). The source of *V. albo-atrum* which incited the 2006 outbreak on farm A is unclear. Future research should clarify dissemination pathways and sources of *V. albo-atrum* to minimize future introductions and spread of the pathogen within and among hop farms.

Although susceptible hop cultivars can still suffer economic damage from nonlethal strains of *V. albo-atrum*, strict attention to limited nitrogen fertilization (20), reduced tillage (19), weed control (19), and proper sanitation of crop residues could be helpful in reducing the incidence and impact of Verticillium wilt in affected yards (15). Several cultivars currently grown in the United States have some level of tolerance to nonlethal strains of *V. albo-atrum* (12,14). Replanting with such cultivars after a long rotation to a non-host is advisable in hop yards where *V. albo-atrum* is established.

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**Literature Cited**