Population Structure of *Verticillium dahliae* Isolates Collected from Potato in the Columbia Basin

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Verticillium dahliae is a soilborne plant pathogen with a worldwide distribution. The fungus causes Verticillium wilt of potato and is a primary component in the potato early dying complex. In North America, vegetative compatibility group (VCG) 4A is found associated with potatoes and exhibits the highest aggressiveness. Primary inoculum of *V. dahliae* is composed of melanized microsclerotia, which have the ability to lie dormant in soils for long periods of time and germinate in response to host root exudates. The fungus invades the roots and colonizes the vascular system of susceptible hosts, causing wilt, chlorosis, necrosis, vascular discoloration, stunting, and premature senescence. During host senescence the fungus produces microsclerotia in colonized host tissue. The disease cycle is completed when infested host debris is incorporated back into the soil. Soilborne primary inoculum levels can increase annually if proper management practices are not observed.

Management of Verticillium wilt aims at reducing primary inoculum through pre-plant fumigation and the use of moderately resistant cultivars and disease-free planting materials. Crop rotation is of only limited benefit due to the wide host range of *V. dahliae*, its ability to colonize and persist on the roots of monocots and other nonhost crops, and its long persistence in soil. Fumigation is expensive and the practice may be eliminated in the future. The use of disease-free planting materials is an important practice, as it reduces the probability of introducing the pathogen or new genotypes (strains) of the pathogen into fields intended for potato production.

The production of potato propagative materials (seed tubers) is restricted to certain seed tubers-producing areas. Seed tubers are shipped to commercial production areas throughout the country annually and the potential exists for long-distance transport of pathogens in planting materials. It was previously shown that inoculum of *V. dahliae* can be found in the vascular system of certified tubers used for seed. Surveys of 224 seed lots intended for U.S. production fields found *V. dahliae* in 29% of the lots and 3.6% of the seed tubers in 1995 and 1996, respectively. All 162 isolates tested belonged to VCG 4, of which 64% belonged to VCG 4A, 33% to VCG 4B and 3% to VCG 4AB. Although *V. dahliae* can be transmitted in certified seed tubers, tuber-borne inoculum (internal infection of the vascular tissue of the seed) appears to have little effect on Verticillium wilt symptoms and potato yields. In a recent study, vascular infection by *V. dahliae* of seed tubers of the moderately susceptible cultivar 'Russet Burbank' resulted in a negligible effect on the development of Verticillium wilt symptoms, did not significantly contribute to aboveground stem infection or the formation of microsclerotia in debris, and did not significantly contribute to progeny tuber infection. However, tuber-borne inoculum may be an important way to introduce inoculum to soils not previously used to grow potato, or where a management practice such as fumigation has been applied to reduce soilborne inoculum.

Another potential source of *V. dahliae* inoculum is field soil associated with seed tubers, either in the form of soil attached to the surface of seed tubers or as loose soil associated with the handling and transport of seed tubers. If infested with *Verticillium*, then this soil could be another source of inoculum in commercial potato fields following fumigation. The purpose of this research was to compare the genetic diversity of *V. dahliae* isolates associated with potato

production in the Columbia Basin. A total of 27 isolates, representing 27 different seed lots from MT and ID in 2008 and 2009, were obtained from infected seed tubers sampled. Seven isolates from infested soil scraped from the surface of seed tubers were obtained from seed lots sampled in 2009. Isolates were characterized by vegetative compatibility group and using mating-type and microsatellite DNA markers. Isolates associated with 2008-2009 seed lots were compared to 15 isolates obtained in 1998 from Columbia Basin (OR, WA) field soils in potato rotations and 30 isolates collected from potato and seed potato in ID, MT, ND, NE, OR, SD, WA, WY.

Vegetative Compatibility Group (VCG) Assay

Chlorate-resistant *V. dahliae* mutants (*nit* mutants) were obtained and paired with known Nit1 and NitM VCG testers on minimal media. Plates were checked for complementation at 2-3 weeks and rated as: no complementation (no reaction), weak complementation (sparse growth of aerial hyphae but no microsclerotia formation), moderate complementation (growth of aerial hyphae and sparse microsclerotia formation), or strong complementation (full wild-type growth, with growth of aerial hyphae and microsclerotia formation). All isolates collected from infected seed tubers and infested soil scraped from seed tubers were assigned to VCG 4 and most (91%) belonged to VCG 4A (Table 1).

Mating-type (*MAT*) Gene Assay

Although the life cycle of *V. dahliae* is considered to be strictly asexual, recent work identifying mating-type (*MAT*) genes in *V. dahliae* revealed the possibility for sexual reproduction of the pathogen (8). Sexual reproduction can contribute to increased genotypic diversity of populations through genetic recombination, which can lead to novel gene combinations and new strains. Isolates of *V. dahliae* were assayed for mating-type genes using a multiplex polymerase chain reaction (PCR). All isolates tested were mating-type *MAT1-2*, indicating that sexual recombination is not likely in populations associated with potato in the Columbia Basin. However, the introduction of the other mating-type through crop rotation or future propagative materials is still a possibility.

Microsatellite Analysis

Eight microsatellite markers, previously shown to be variable in *V. dahliae* populations collected from lettuce, strawberry and other hosts from coastal California and Wisconsin (1), were used in the analysis. Fifteen additional isolates from infested soil and 30 isolates from infected potato obtained in other states were included for comparison. Genotyping was performed using a nested-PCR reaction to fluorescently label PCR products, essentially as described by Schuelke (5) and products were separated on an ABI 3100 sequencer. Genetic diversity (4) and Slatkins R_{st} (6) were calculated using the software Arlequin ver. 3.5 (2). An R_{ST} value of 0 indicates no separation; $0 < R_{ST} < 0.05$ indicates negligible differentiation; $0.05 \leq R_{ST} < 0.25$ indicates moderate differentiation; $R_{ST} \geq 0.25$ indicates high differentiation; and $R_{ST} = 1$ indicates complete differentiation. Analysis of molecular variance (AMOVA) was performed using Arlequin to test for genetic differentiation between VCG 4A and VCG 4B subgroups affecting potato. Isolates were divided into two clusters (VCG 4A and VCG 4B) with four sample populations in each (infected seed tubers, infested soil from seed tubers, Columbia Basin field soils, and potatoes and seed potatoes grown in ID, MT, ND, NE, OR, SD, WA, WY). Genetic distances were calculated using Genotype (3) and a minimum spanning network was constructed from genetic distances using HapStar ver. 0.6 (7).

A total of 14 microsatellite haplotypes (haploid genotypes, or strains) were found among the 80 isolates collected from hosts associated with potato production (seed tubers, soil scraped from seed tubers, infested field soils, and infected potato plants from various states). The potato group exhibited the greatest genetic diversity as indicated by the relative number of haplotypes to isolates sampled (Table 1), followed by infested soil scraped from seed tubers and infested field soils. A single haplotype was predominant in all VCG 4A sample populations. This haplotype made up 100% of VCG 4A isolates from seed tubers and 67% of VCG 4A isolates from infested soil scraped from seed tubers. This haplotype also accounted for 80% of VCG 4A isolates from infested field soils.

Over 63% of the genotypic variability was observed among VCG sampling groups ($P < 0.02857$), indicating significant differentiation among VCGs. Only 3% ($P = 0.17163$) of variability was explained by differences among sample populations (seed tubers, soil scraped from seed tubers, infested field soils, and potato from various states), while the remaining variability (approximately 33%) was explained by genetic diversity within sample populations ($P < 0.00001$). All VCG 4A sample populations were highly differentiated from VCG 4B sample populations ($R_{st} > 0.5$) (Table 2). Although minimum spanning network analyses differentiated two general clusters corresponding to VCG 4A and VCG 4B subgroups, some VCG 4A isolates appear to be more closely related to VCG 4B and VCG 4A/B isolates than to other VCG 4A isolates (Fig. 1).

Conclusions

The amount and distribution of genetic diversity in plant pathogen populations can directly impact disease epidemiology and management efficacy. Greater genetic and genotypic variability is often associated with an increased ability to adapt to changing environments and selective pressures. Sexual recombination increases genotypic variability by redistributing existing genetic variability into new combinations, or genotypes. All isolates characterized in this study possessed the *MAT1-2* idiomorph, indicating that the potential for sexual recombination among these populations is low.

Genotypic diversity varied among sample groups, ranging from a nearly clonal population in potato seed tubers to a relatively diverse population in potato and Columbia Basin field soils. The diversity found among potato isolates is likely due to the various states sampled, while the genetic diversity observed in the Columbia Basin field soil sample groups may be due to differences in the cropping histories of the fields. Most isolates (93%) obtained from infected seed potato were a single haplotype, indicating that genotypic diversity of *V. dahliae* isolates in seed lots is low. However, the genotypic diversity of *V. dahliae* isolates found in tare soil attached to seed tubers is relatively high. This source of inoculum may be especially important, since levels of *V. dahliae* in seed tare soil can be greater than 500 CFU/g soil (*data not shown*). Although the contribution of seed tare soil to Verticillium wilt is not known, even a small amount of infested seed tare soil (1 g soil/metric ton of seed) may be significant given the large quantity of seed (approximately 150,000 metric tons) planted in the Columbia Basin every year.

The potential exists for the exchange of heritable material between vegetatively compatible but genetically distinct isolates of *V. dahliae*. The majority of isolates characterized in this study were assigned to VCG 4A, which has previously shown to be highly aggressive on potato. The remaining isolates assigned to either VCG 4B or VCG 4A/B, which is also pathogenic on potato but to a lesser extent than VCG 4A. AMOVA and Slatkin's R_{st} values indicated that the genetic differentiation between VCG 4A and 4B subgroups was significant.

However, both subgroups contained a significant proportion of the total genetic variation, indicating genetic diversity exists within VCG 4A and VCG 4B. Although VCG 4 subgroups are genetically differentiated, the potential may exist for the exchange of genetic material between strains of VCG 4A and VCG 4B via VCG 4A/B strains.

A single microsatellite haplotype was associated with VCG 4A populations from all four sample groups and was predominant in infected seed potato. This haplotype was found in potato and field soils in rotation with potato in several states (WA, OR, SD, WY, ND, and NE), indicating a wide distribution. Several isolates of this haplotype were previously demonstrated to be highly aggressive on potato in greenhouse assays (*data not shown*). This haplotype was also isolated from *V. dahliae*-infected raspberry and blackberry (*Rubus*), watermelon (*Citrullus*), sugar beet (*Beta*), cherry (*Prunus*), and maple (*Acer*) growing in ID, OR, and WA. The exact origin of this particular haplotype is currently not known. A better understanding of the population structure of *V. dahliae* will increase the potential to better manage Verticillium wilt of potato in the future.

References

1. Atallah, Z. K., Maruthachalam, K., Toit, L. d., Koike, S. T., Michael Davis, R., Klosterman, S. J., Hayes, R. J., and Subbarao, K. V. 2010. Population analyses of the vascular plant pathogen *Verticillium dahliae* detect recombination and transcontinental gene flow. Fungal Genetics and Biology 47:416-422.
2. Excoffier, L., Laval, G., and Schneider, S. 2005. Arlequin (version 3.0): An integrated software package for population genetics data analysis. Evolutionary Bioinformatics Online 1:47-50.
3. Meirmans, P. G., and Van Tienderen, P. H. 2004. GENOTYPE and GENODIVE: Two programs for the analysis of genetic diversity of asexual organisms. Molecular Ecology Notes 4:792-794.
4. Nei, M. 1973. Analysis of gene diversity in subdivided populations. Proceedings of the National Academy of Sciences of the United States of America 70:3321-3323.
5. Schuelke, M. 2000. An economic method for the fluorescent labeling of PCR fragments. Nature Biotechnology 18:233-234.
6. Slatkin, M. 1995. A measure of population subdivision based on microsatellite allele frequencies. Genetics 139:457-462.
7. Teacher, A. G. F., and Griffiths, D. J. 2010. HapStar: automated haplotype network layout and visualization. Molecular Ecology Resources.
8. Usami, T., Itoh, M., and Amemiya, Y. 2009. Asexual fungus *Verticillium dahliae* is potentially heterothallic. Journal of General Plant Pathology 75:422-427.

Table 1. Sample groups, vegetative compatibility group (VCG), the number of individuals, and the number of haplotypes (strains) found in *V. dahliae* populations used in this study.

Sample group	VCG	No. of isolates	No. of haplotypes
Infected seed tubers[a]	4A	25	1
	4B	2	1
	Total	**27**	**2**
Infested seed surface soil[b]	4A	7	3
	4B	1	1
	Total	**8**	**3**
Columbia Basin field soils[c]	4A	11	4
	4B	3	2
	4A/B	1	1
	Total	**15**	**6**
Potato from various states[d]	4A	15	7
	4B	12	7
	4A/B	3	2
	Total	**30**	**12**

[a] Isolates from infected seed tubers were collected from seed lots intended for Columbia Basin production fields between 2007 and 2009.
[b] Isolates from infested soil on the surface of seed tubers were obtained from seed lots intended for Columbia Basin production fields in 2009.
[c] Isolates from Columbia Basin field soils were collected in 1998 from soils in OR and WA associated with potato production.
[d] The potato sample group consisted of isolates collected from infected potato and seed potato in various states (ID, MT, ND, NE, OR, SD, WA, WY) during the mid-1980's and 1990's.

Table 2. Gene diversity differentiation of *V. dahliae* VCG 4A and VCG 4B sample populations as indicated by Slatkin's R_{ST}. An R_{ST} value of 0 indicates no separation; $0 < R_{ST} < 0.05$ indicates negligible differentiation; $0.05 \leq R_{ST} < 0.25$ indicates moderate differentiation; $0.25 \leq R_{ST} < 1$ indicates high differentiation; and $R_{ST} = 1$ indicates complete differentiation.

	Sample population (VCG 4A)			
Sample population (VCG 4B)	**Infected seed tubers**	**Infested seed surface soil**	**Columbia Basin field soils**	**Potatoes**
Infected seed tubers[a]	1.00000	0.82090	0.78454	0.99648
Infested seed surface soil[b]	1.00000	0.64444	0.54571	0.99043
Columbia Basin field soils[c]	1.00000	0.81028	0.75368	0.99589
Potato from various states[d]	0.89969	0.66116	0.62440	0.73801

[a] Isolates from infected seed tubers were collected from seed lots intended for Columbia Basin production fields between 2007 and 2009.
[b] Isolates from infested soil on the surface of seed tubers were obtained from seed lots intended for Columbia Basin production fields in 2009.
[c] Isolates from Columbia Basin field soils were collected in 1998 from soils in OR and WA associated with potato production.
[d] The potato sample group consisted of isolates collected from infected potato and seed potato in various states (ID, MT, ND, NE, OR, SD, WA, WY) during the mid-1980's and 1990's.

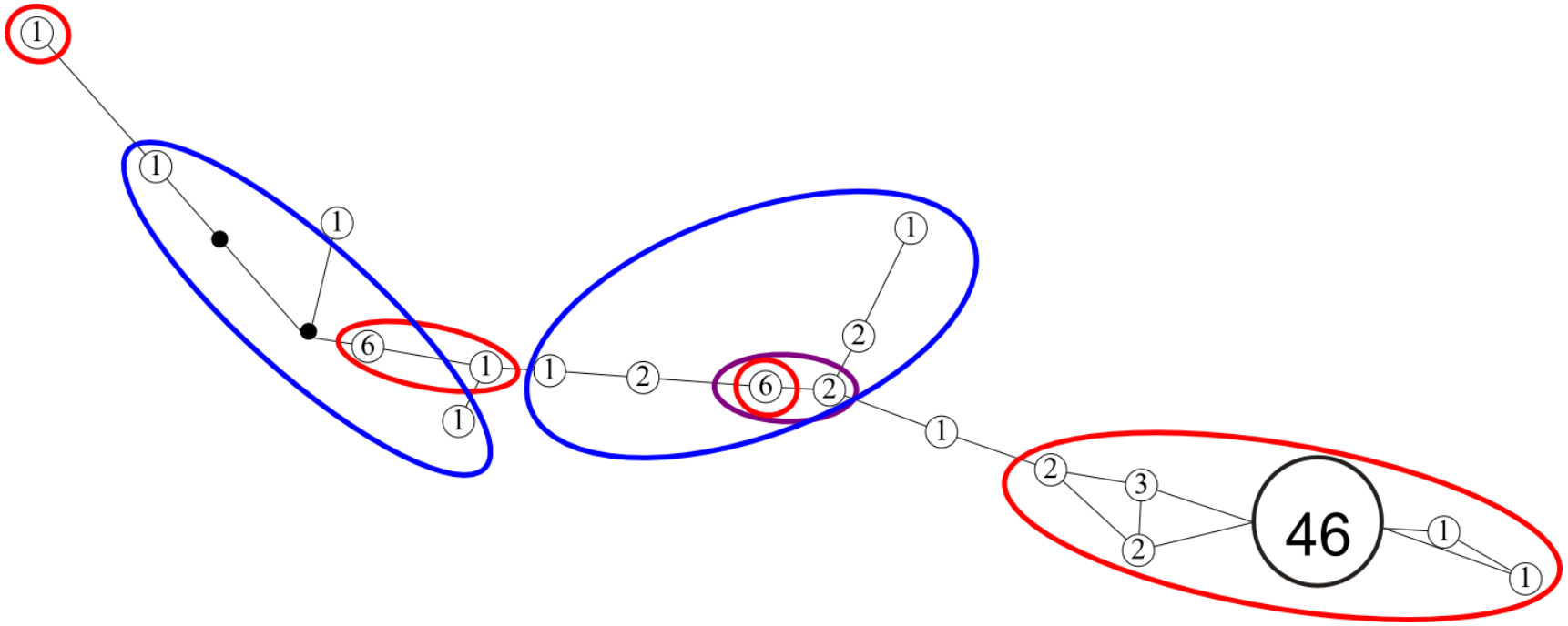

Fig. 1. Minimum spanning network showing the relationships between microsatellite haplotypes of *V. dahliae* collected from infected seed tubers, infested soil scraped from seed tubers, Columbia Basin (OR,WA) field soils, and infected potato plants and seed from various states (ID, MT, ND, NE, OR, SD, WA, WY). Numbers within circles indicate the number of isolates sampled per haplotype. Colored circles indicate the vegetative compatibility groups (VCGs) of haplotypes (red: VCG 4A; blue: VCG 4B; purple: VCG 4A/B; no circle: not defined)