

Experimental evolution reveals temperature-dependent phenotypic and genomic changes in *Verticillium alfalfae*, the causative agent of alfalfa Wilt

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Motivation and aim: *Verticillium* wilt is a destructive soil-borne disease affecting diverse crops from vegetables to trees, causing severe yield losses and reducing the quality of agricultural products. The disease is caused by various *Verticillium* fungal species, which differ in host specificity and environmental adaptability. Among these, *Verticillium alfalfae* is a key pathogen of the major forage crop, alfalfa (*Medicago sativa*), and its wild relative *Medicago truncatula*. Our previous studies revealed that *V. alfalfae* exhibits remarkable thermal adaptation capabilities, with temperature shifts significantly influencing its virulence (Sbeiti et al., 2023). To further investigate this phenomenon, we conducted an artificial evolution experiment, generating a collection of warm-temperature-adapted mutant strains. The primary objective of this study is to characterize the phenotypic and genetic diversity within this adapted collection, providing insights into the mechanisms underlying thermal adaptation and its impact on virulence.

Materials and methods: Wild-type *V. alfalfae* strain was exposed to UV irradiation and selectively propagated for enhanced pathogenicity into increased temperature condition using a susceptible *M. truncatula* genotype three times. After third round of mutagenesis monosporal temperature-adapted mutants were isolated and phenotyped for virulence across *M. truncatula* accessions with varying resistance levels. From these, a set of 23 mutant strains with different virulence was established for in-depth phenotypic characterization, including morphological traits (colony growth, sporulation rate) and extracellular enzymatic activities linked to virulence (lipase, protease, cellulase, polygalacturonase). To identify genetic underpinnings of observed diversity, whole-genome sequencing was performed on the 5 most contrasting mutant strains using a dual-platform approach (Oxford Nanopore for long-read; Illumina for short-read). High-quality genomes were analyzed for SNPs and candidate mutations associated with thermal adaptation and virulence.

Results: The selected 23 representative mutant strains (plus wild type) showed significant variation in sporulation rate and extracellular enzymatic activity, particularly in cellulase and polygalacturonase production. Whole-genome sequencing of four contrasting mutants and the wild type using a dual-platform approach (Illumina/Oxford Nanopore) generated high-quality assemblies, enabling identification of SNPs in genes related to fungal metabolism and vesicular transport that likely contribute to the observed thermal adaptation and virulence changes.

Conclusions: Our study demonstrates that temperature elevation acts as an evolutionary force, driving significant alterations in fungal virulence. Through comprehensive phenotypic characterization of warm-adapted *V. alfalfae* mutants, we identified distinct profiles in morphological traits (colony growth, sporulation rate) and virulence-related enzymatic activities (lipase, protease, cellulase, polygalacturonase). Whole-genome analysis revealed candidate SNPs potentially responsible for these adaptive changes, providing the first genetic insights into thermal adaptation in this pathogen. These findings establish a critical foundation for predicting how climate change may influence *Verticillium* wilt epidemiology. Moving forward, we will expand our analysis to the complete collection of 180 temperature-adapted strains, enabling genome-wide association studies to systematically identify genomic loci underlying thermal adaptation and virulence – key knowledge for developing climate resilient crop protection strategies.

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