Pseudomonas SPECIES CAUSING DISEASES IN ALFALFA*

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Review paper

Abstract

The *Pseudomonas* genus includes species that are significant pathogens, causing considerable losses in plant production. Many of these pathogens lead to diseases that have global economic and environmental consequences for the trade of plants, seeds, and food. Alfalfa (*Medicago sativa*) is one of the most widely cultivated and important forage crops in the world. Its exceptional qualities, such as high biomass yield, excellent forage quality, and preference among ruminants, have earned it the nickname "Forage Queen." However, certain phytopathogenic species of *Pseudomonas* can cause substantial yield losses in alfalfa production. This review discusses the diseases caused by *Pseudomonas* species in alfalfa, their symptoms, and the mechanisms through which these pathogens cause disease. It also covers techniques for detecting and isolating these species from plants and soil, as well as methods for controlling these diseases. This review aims to provide researchers with comprehensive scientific information on managing *Pseudomonas*-related diseases in alfalfa production.

Keywords: *Pseudomonas, Alfalfa, Bacterial diseases, Medicago sativa*

INTRODUCTION

Alfalfa (*Medicago sativa*), often referred to as the "Queen of Forages," is a perennial leguminous crop that has been cultivated for over 2,000 years (Chen *et al*., 2020). It is a vital forage crop worldwide, especially for livestock production, and holds significant importance for dairy farmers. Furthermore, alfalfa plays a critical role in sustainable agricultural systems. Its benefits include high biomass yield, contributions to soil and water conservation, enhancement of soil fertility through biological nitrogen fixation, suppression of pests and pathogens in crop rotations, and provision of habitat for wildlife (Putnam *et al*., 2001).

Plant pathogens and nematodes that infect alfalfa can significantly reduce forage yield and quality, alongside shortening the plant's lifespan (Nemchinov *et al*., 2017). Most research on alfalfa diseases has traditionally focused on fungal infections (Zhibiao, 1985), while studies on bacterial diseases affecting alfalfa remain relatively limited. To

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date, nine bacterial diseases have been reported in alfalfa. The bacteria responsible for these diseases belong to several genera, including *Erwinia*, *Pectobacterium*, *Agrobacterium*, *Pseudomonas*, *Xanthomonas*, *Clavibacter*, and *Xylella*. Within the genus *Pseudomonas*, two species have been documented as pathogens of alfalfa: *Pseudomonas syringae* pv. *syringae* and *Pseudomonas viridiflava* (Gray & Hollingsworth, 2015).

This review focuses on bacterial diseases affecting alfalfa globally, specifically addressing the distribution and host range of diseases caused by pathogens from the *Pseudomonas* genus. It also offers a comprehensive overview of the symptoms and identifying characteristics of these pathogens, summarizes common diagnostic methods, and discusses effective strategies for disease control.

Bacterial stem blight (*Pseudomonas syringae* pv. *syringae*)

Alfalfa bacterial stem blight (BSB) was first identified in 1904 in Colorado, where frost damage was noted as a potential entry point for the pathogen (Sackett, 1910). This disease is widespread in the central and western regions of the United States and has also been reported in Australia, the United Kingdom, the former Yugoslavia, Russia, and Iran. Although it usually results in minimal losses, it can cause 40-50% loss of forage during the first harvest in some high valleys in the western United States (Nyvall, 2013).

Symptoms of the Disease: Alfalfa bacterial stem blight typically begins in early spring and persists until the first harvest. The symptoms include necrotic lesions on the stems, chlorosis on the leaves, bending of the stems in a shepherd's crook shape, and water-soaked spots (Gray & Hollingsworth, 2015; Samac *et al*., 2014). These spots transition from light brown to black and spread toward the roots (Nyvall, 2013). Infected stems become thin, brittle, and shorter than healthy plants. While vascular tissues remain unaffected, the leaves turn yellow and eventually wither (Zhenfan & Zhibiao, 2014).

Causal Agent: The causal agent is *Pseudomonas syringae* pv. *syringae*. This bacterium is a gram-negative, motile rod with rounded ends, measuring 0.5–0.8 × 1.2–2.4 µm. Each end has one to four flagella, and the bacteria may form long chains. Colonies grown on nutrient agar appear smooth, circular, slightly convex, grayish-white, glistening, and translucent. They also produce a greenish fluorescent pigment that diffuses into the medium. The optimal temperature for growth is between 27 and 30°C. The host range for *P. syringae* strains found in alfalfa is not well-defined, but it's common for isolates within this group to have overlapping host ranges (Nyvall, 2013). *P. syringae* has two main phases: epiphytic and endophytic, and environmental factors influence its pathogenic potential in both phases (Xin *et al*., 2018).

Disease Cycle and Epidemiology: In spring, cold and humid conditions create an environment that is conducive to disease development in alfalfa. *Pseudomonas syringa*e pv. *syringae* contributes to frost damage through ice nucleation activity and can survive in plant residues in the soil. It typically enters the plant through areas that have been affected by frost. Under favorable environmental conditions, the disease can spread

within the field, and it is generally observed during the first harvest in the United States (Nyvall, 2013).

Infection Mechanism of the Pathogen: *Pseudomonas syringae* employs several virulence factors to establish infection, including the Type III secretion system (T3SS), ice nucleation activity, toxins, cell wall-degrading enzymes, and exopolysaccharides (Morris *et al*., 2013). This pathogen, specifically *P. syringae* pv. *syringae*, infects its host primarily through frost damage, with ice nucleation proteins (INPs) located in its outer membrane contributing to this process (Li *et al*., 2012). The disease progression occurs in two stages: localized leaf necrosis (or blight) and systemic vascular wilting. In the first stage, water-soaked lesions, resulting from frost injuries, spread along the stem, leading to dried bacterial exudates. The ice nucleation capability of *P. syringae* exacerbates frost damage, causing significant economic losses, particularly in plants that are vulnerable to late spring frosts (Lindow *et al*., 1982).

Management of Bacterial Stem Blight (BSB) Disease: Currently, there is no reliable method to predict BSB disease in advance, nor is there an effective chemical control available. Given the lack of commercially resistant alfalfa varieties, the importance of integrated management strategies is increasing (Baltrus *et al*., 2017; Yu & Kole, 2021). Cultural control methods, such as selecting frost-resistant alfalfa varieties and harvesting early after frost, can help reduce disease spread by eliminating infected plant material (Nemchinov *et al*., 2017). While copper-based bactericides have shown limited success in managing the disease (Scheck *et al.,* 1998), biological control options are emerging. Antagonistic bacteria like *Pantoea agglomerans* and *Bacillus* species show promising results, and natural antibacterial compounds, along with thermal seed treatments, also present alternative solutions (Balestra & Bovo, 2003). Genetic research is ongoing to develop BSB-resistant varieties. Although the ZG9830 variety has been identified as a potential candidate for resistance, its low yield poses a significant challenge (Nemchinov *et al*., 2017). Therefore, employing resistant varieties in conjunction with integrated management strategies is the most sustainable approach to mitigating the impacts of BSB.

Bacterial crown and root rot (*Pseudomonas viridiflava*)

This disease, commonly observed in many alfalfa production areas worldwide, was first described by Burkholder in 1930 and was recognized as a primary limiting factor in alfalfa production by Dowson in 1939 (Dowson, 1939).

Symptoms of the Disease: In the field, symptoms of the disease manifest as light brown, dry rot on the crown and main taproot. Light brown streaks extend beyond the rot, spreading through the vascular system along approximately one-third of the root (Nyvall, 2013). In affected plants, symptoms include yellowing, loss of green color, and wilting. This is often followed by further wilting, necrosis, stunted growth, and leaf deformation (Zhenfan & Zhibiao, 2014).

Causal Agent: The causative agent of the alfalfa bacterial crown and root rot complex is *Pseudomonas viridiflava*. This rod-shaped bacterium measures 0.5–1.0 μm in width and 1.5–5.0 μm in length. As a Gram-negative organism, it moves using a polar

flagellum. On media containing 5% sucrose, its colonies typically appear yellow, whereas on media with yeast extract and glycerol, the colonies can range from olive green to golden yellow. Additionally, some strains are capable of producing a blue pigment (Heydari *et al*., 2012).

Disease Cycle and Epidemiology: The initial infection of the disease occurs when bacteria enter the roots through wounds, infection sites, nematode feeding marks, winter injuries, or mechanical damage. Additionally, newly cut stems during harvest can serve as entry points for bacteria. The disease spreads most rapidly under moderate temperatures and moist soil conditions. Bacteria can survive in plant material, soil, and straw, and they can be transmitted from plant to plant through sprinkler irrigation or contaminated agricultural tools (Heydari *et al*., 2014).

Infection Mechanism of the Pathogen: This pathogen spreads through the xylem and obstructs water transport, leading to symptoms such as stunted growth, chlorosis, and wilting in plants. It produces exopolysaccharides that block water flow, resulting in root rot, yellowing of leaves, and large lesions on the stems and roots. The pathogen also breaks down plant tissues using various enzymes, including cutinase, pectinase, cellulase, protease, and hemicellulase (Murillo & Sesma, 2001). Infected plants exhibit color changes in their roots, shifting from yellow to brown, along with clustering of small leaves and upward curling of leaves. In severe cases, this can ultimately lead to plant death (Heydari *et al*., 2014).

Management of the Disease (Bacterial Crown and Root Rot): Although there is currently no effective management method for diseases caused by *Pseudomonas viridiflava*, the application of *Bacillus* species and *Pseudomonas fluorescens* for biological control and the use of copper compounds (such as Bordeaux mixture, copper hydroxide, and copper oxide) have shown promising results in managing epiphytic populations (Balestra & Bovo, 2003; Fascella *et al*., 2015; Al-Karablieh *et al.,* 2017). Genetic studies are focused on understanding the pathogenic characteristics of *P. viridiflava* and on developing resistance genes (Bartoli *et al*., 2015). Early diagnostic techniques, such as PCR, can help limit the spread of the disease, although no specific early detection method currently exists (Bull & Koike, 2015).

Taxonomy of *Pseudomonas* species causing disease in alfalfa

The *Pseudomonas* genus is a highly diverse group of Gram-negative bacteria that can be phylogenetically divided into two main lineages: the *P. fluorescens* lineage and the *P. aeruginosa* lineage. The *P. fluorescens* lineage includes most plant-associated species, including significant phytopathogens like *P. syringae*. *P. syringae* is recognized as a complex species group that consists of nine genomospecies, 13 phylogroups, and over 60 pathovars, many of which are responsible for diseases in various plant hosts (Bull *et al.,* 2010; Morris *et al*., 2008). Another important member of this lineage is *P. viridiflava*, which falls within phylogroups PG7 and PG8 and is characterized by distinct pathogenic traits and adaptability to different environments (Parkinson *et al*., 2011).

Pseudomonas syringae is a significant phytopathogen that has been extensively studied for its genomic and phylogenetic classifications. This bacterium causes various plant

diseases and is classified using techniques such as single-gene sequencing, multilocus sequence analysis (MLSA), multilocus sequence typing (MLST), repetitive extragenic palindromic PCR (rep-PCR), and whole-genome sequencing (Berge *et al.,* 2014). *P. syringae* belongs to the *Pseudomonas fluorescens* lineage, which contains most plant pathogens (Bull *et al*., 2010). Although *P. syringae* is often treated as a single species, it represents a complex of genetically diverse species comprising nine genomospecies, 13 phylogroups, and 64 pathovars infecting a wide range of hosts (Morris *et al*., 2008). Among these, *P. syringae* pv. *syringae*, which causes bacterial stem blight in alfalfa, is classified in phylogroup 2b (PG2b) within PG2, and is found widely in agricultural and natural environments (Morris *et al*., 2008). PG2 strains are recognized for their pathogenicity and virulence factors, including toxin production, type III secretion systems (T3SS), and quorum sensing; they often shift from epiphytic to endophytic phases (Xin *et al*., 2018). In contrast, *Pseudomonas viridiflava*, which causes bacterial crown and root rot, falls under phylogroups PG7 and PG8. Research has shown that these phylogenetic groups differ in pathogenicity and environmental adaptability, with PG2 strains generally being more aggressive in agricultural settings. The taxonomic complexity of *P. syringae* and *P. viridiflava* has led to the utilization of various genes, such as *gyrB*, *rpoD*, and *16S rRNA*, for species identification (Yamamoto *et al*., 2000). Additional housekeeping genes, including *gapA*, *cts*, and *purA*, further enhance the classification of *P. viridiflava* isolates using MLSA and single-gene analyses (Parkinson *et al*., 2011). Recent molecular methods, such as rep-PCR and MLSA, have proven effective in the precise identification of these pathogens (Bull & Koike, 2015).
Future research on the virulence and genetic diversity of *P. syringae* and *P. viridiflava* will be essential for disease management and crop loss reduction, particularly in alfalfa and similar agricultural crops.

Common diagnostic methods for identifying *Pseudomonas* species that cause diseases in alfalfa

The most important factor in fighting diseases caused by *Pseudomonas* species and in developing resistance is the precise identification of the pathogen (Guilbaud *et al*., 2016). Accurate diagnosis of *Pseudomonas* species that affect alfalfa is achieved through various methods, including culture-based techniques, PCR methods, molecular fingerprinting, and biochemical tests (Parisi *et al*., 2019).

Culture-Based and Biochemical Diagnostic Methods for *Pseudomonas* Species: Traditional culture-based methods are commonly used to diagnose *Pseudomonas* species. King's B medium is effective for isolating species that exhibit green fluorescence under ultraviolet light (Schaad *et al*., 2001).
The LOPAT test—comprising a series of determinative tests: L for levan production, O for oxidase production, P for pectinolitic activity, A for arginine dihydrolase production, and T for tobacco hypersensitivity - provides an accurate diagnosis by distinguishing between pathogenic and non-pathogenic fluorescent *Pseudomonas* species. However, it

has limitations when it comes to differentiating between strains and pathovars (Goudarzi & Mortazavi, 2020).

Biochemical tests are conducted to assess the biological characteristics of *Pseudomonas* strains. Notably, ice nucleation activity allows *P. syringae* strains to damage plant tissue at low temperatures, which can increase disease severity (Bull & Koike, 2015). Furthermore, biochemical analysis of compounds associated with virulence factors, such as lipoproteins, mono-oxygenase, and various polysaccharides, is a valuable tool in diagnosis (Bartoli *et al.,* 2015).

PCR-Based Diagnostic Methods: DNA-based techniques, especially the amplification of 16S and 23S rRNA genes via PCR, are commonly utilized to identify various *Pseudomonas* species and assess their genetic diversity (Olczak-Woltman *et al.,* 2007). In the case of *P. syringae*, the *syrB* gene serves as a marker for identifying syringomycin production. Additionally, species-specific primers targeting lipoprotein and monooxygenase genes are employed for diagnostic purposes in multiplex PCR for *P. viridiflava* (Lipps *et al*., 2019).

DNA Sequencing and Phylogenetic Analysis of *Pseudomonas* Species: Multilocus sequence analysis (MLSA) and other gene sequencing methods allow for the rapid and accurate identification of *Pseudomonas* species that cause diseases in alfalfa (Berge *et al*., 2014). By using conserved genes such as 16S rRNA, *gyrB*, and *rpoD*, researchers can reliably analyze interspecies relationships (Yamamoto *et al*., 2000). MLSA serves as an effective tool for identifying pathovars within the *Pseudomonas syringae* complex and for examining strain diversity (Parisi *et al*., 2019). The genetic data obtained from these analyses are utilized to construct phylogenetic trees, which help us understand the evolutionary relationships and the diversity of pathogenicity factors in complex species like *P. syringae* and *P. viridiflava* (Bull & Koike, 2015).

Genomic and Molecular Analyses in *Pseudomonas* Species: With the recent widespread adoption of whole-genome sequencing methods, researchers are closely examining the genetic differences among various pathovars of *Pseudomonas syringae* and the evolutionary conservation of factors related to pathogenicity. These analyses provide valuable insights into horizontal gene transfer, the diversity of effector proteins, and adaptations to different environments, significantly enhancing our ability to detect and classify pathogens (Parisi *et al*., 2019). Molecular fingerprinting techniques, such as rep-PCR, are particularly effective for the intraspecific identification of pathogenic species like *P. syringae* and *P. viridiflava*, and they play a crucial role in monitoring disease outbreaks (Bull & Koike, 2015).

CONCLUSİONS

Two species of the genus *Pseudomonas* cause alfalfa (*Medicago sativa*) diseases, resulting in serious issues such as yield losses and reduced plant lifespan. *Pseudomonas syringae* and *Pseudomonas viridiflava* play a pivotal role in the spread of these diseases. This study provides critical insights into the disease mechanisms, infection processes, and impacts of these *Pseudomonas* species on plants, contributing valuable information for developing effective management strategies for these pathogens.

In conclusion, developing resistant alfalfa cultivars and advancing biological control methods could offer long-term, sustainable solutions for combating these bacterial pathogens. Additionally, the broader adoption of modern diagnostic techniques for detecting and monitoring the spread of bacterial diseases will be an essential step in disease management. In this context, the presented findings hold significant importance for scientific research and agricultural applications.

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