Plant Protection, 09 (04) 2025. 1067-1079

DOI:10.33804/pp.009.04.5986

Available Online at EScience Press

Plant Protection

ISSN: 2617-1287 (Online), 2617-1279 (Print)

http://esciencepress.net/journals/PP

Research Article

Geospatial Mapping and Biochemical Characterization of Walnut Bacterial Blight Caused by *Xanthomonas arboricola* pv. *juglandis* in the Temperate Regions of Azad Jammu and Kashmir, Pakistan

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ARTICLE INFO

Article history
Received: 29th September, 2025
Revised: 22nd December, 2025
Accepted: 28th December, 2025

Keywords
Walnut bacterial blight
Xanthomonas arboricola pv. juglandis
Disease surveillance
Percent disease index
Azad Jammu and Kashmir

ABSTRACT

Bacterial blight of walnut (*Juglans regia* L.), caused by the Gram-negative bacterium *Xanthomonas arboricola* pv. *juglandis*, has recently emerged as a serious threat to walnut production in the temperate regions of Azad Jammu and Kashmir (AJK), Pakistan. The present study provides the first comprehensive surveillance and biochemical characterization of walnut bacterial blight across major walnut-producing districts of AJK. Systematic field surveys were conducted in eight districts, with ten locations surveyed per district, to assess disease prevalence, incidence, and severity on walnut leaves and fruits using a standardized 0-4 disease rating scale. The mean percent disease index (PDI) across the region ranged from 69.00% to 76.63%, indicating moderate to very high disease pressure. Disease prevalence varied between 60% and 100%, reflecting widespread distribution of the disease, while disease incidence reached 100% at several locations. The pathogen was consistently isolated from symptomatic tissues and produced characteristic yellow, mucoid colonies on nutrient agar and yeast dextrose calcium carbonate media. Biochemical characterization revealed typical reactions of *Xanthomonas* spp., including a Gram-negative reaction, positive catalase activity, and a negative oxidase test. Pathogenicity assays conducted on walnut leaflets and immature fruits successfully reproduced typical blight symptoms, and Koch's postulates were fulfilled through re-isolation of the pathogen. Overall, the findings demonstrate severe disease pressure in the region and confirm *X. arboricola* pv. *juglandis* as the primary causal agent of walnut bacterial blight in AJK. This study provides a critical baseline for future epidemiological investigations and supports the development of integrated, region-specific disease management strategies.

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Introduction

The walnut (*Juglans regia* L.) is a temperate nut crop widely cultivated in Central Asia, China, Europe, the USA, and South Asia. It is important both economically and nutritionally, supporting rural livelihoods, agroforestry practices, and specialized export markets for dried fruits in Pakistan, particularly in the temperate and sub-temperate regions of Azad Jammu and Kashmir (AJK).

Walnut production in AJK benefits from the region's favorable climate, characterized by cool temperatures and high spring humidity. However, these same conditions also increase the vulnerability of walnut orchards to several diseases, with bacterial blight being the most destructive.

Bacterial blight, caused by *Xanthomonas arboricola* pv. *juglandis* (*Xaj*), is the most severe disease affecting walnuts globally. Fruit infection can cause early fruit drop and substantial yield losses, sometimes exceeding 70% under favorable environmental conditions (Lamichhane, 2014). Epidemics are closely associated with persistent rainfall, high relative humidity, and mild temperatures during flowering and early fruit development. *Xaj* is genetically diverse, exhibiting significant regional variation in virulence, host interaction, and epidemiological behavior. Numerous studies have shown that *Xaj* genotypes differ considerably at both phenotypic and molecular levels, influencing disease severity and treatment outcomes (Kałużna et al., 2021; Ji et al., 2025).

Molecular approaches, including PCR-based assays and phylogenetic analyses, have become essential for reliable pathogen identification, demonstration of pathogenicity, and assessment of genetic diversity among *Xaj* populations (Weisburg et al., 1991; Tamura et al., 2013; Essakhi et al., 2015). Understanding this diversity is critical for developing location-specific management strategies, breeding resistant varieties, and predicting disease outbreaks. Despite its global importance, walnut bacterial blight remains understudied in Pakistan. In AJK, one of the country's main walnut-producing regions, the prevalence of the disease, distribution, and severity have not been extensively documented, and the pathogen has not been thoroughly characterized morphologically, biologically, or genetically. The lack of epidemiological data and understanding of pathogen diversity limits the development of effective control measures and policy-level interventions.

Integrating field surveillance with molecular characterization provides reliable information on pathogenic biodiversity and phylogenetic relationships, particularly in understudied regions such as AJK. Phylogenetic studies of *Xaj* genotypes also support local and international disease monitoring by revealing patterns of pathogen adaptation, distribution, and potential introduction pathways. This study aimed to conduct a comprehensive survey of walnut-producing areas in AJK to determine the prevalence, incidence, and severity of bacterial blight and to isolate and confirm the pathogenicity of *Xaj* using morphological and biochemical analyses. The findings provide essential baseline information for disease risk assessment, sustainable management strategies, and future resistance breeding programs. This represents the first comprehensive investigation of walnut bacterial blight in Pakistan, particularly in AJK.

Materials and Methods

Design and area of the survey

A comprehensive field survey was conducted during the active growing season, from March to May 2023, in the major walnut-producing temperate regions of AJK, Pakistan. The survey encompassed eight districts: Neelum, Jhelum, Muzaffarabad, Bagh, Haveli, Poonch, Sudhnoti, and Kotli, which represent the primary walnut production zones of the area. Ten locations per district were randomly selected based on walnut cultivation density, accessibility, and geographic distribution, resulting in a total of 80 surveyed sites.

At each location, a random sampling approach was employed to assess walnut plantations. Twenty walnut trees were selected at random and examined for symptoms of bacterial blight, including necrotic patches, water-soaked lesions, tissue blackening, and premature fruit decay. Disease assessment was performed on both leaves and immature fruits, which are highly susceptible to infection by *Xaj*. All visual observations were carefully recorded to ensure consistency across all surveyed sites.

Disease severity was quantified using a 0-4 numerical rating scale (Table 1), where higher values indicated increased disease progression, based on lesion size and tissue penetration. The Percent Disease Index (PDI) was calculated using the following formula:

$$\text{PDI} = \frac{\sum \text{of all disease ratings}}{\text{Total number of observations} \times \text{Maximum disease rating}} \times 100$$

Using the following formula, disease incidence was determined as the proportion of infected plants to all plants evaluated at each location:

$$\text{Disease Incidence (\%)} = \frac{\text{Number of diseased plants}}{\text{Total number of plants observed}} \times 100$$

The percentage of assessed locations exhibiting bacterial blight symptoms was used to calculate disease prevalence at district level using the following formula:

$$\text{Disease prevalence (\%)} = \frac{\text{Number of infected locations}}{\text{Total locations surveyed}} \times 100$$

The obtained field information gave a thorough picture of the spatial distribution and severity of bacterial blight in walnut throughout various districts of AJK, and served as the foundation for further pathogen isolation, characterization, and molecular identification (Nutter et al., 2006; Lamichhane, 2014).

Table 1. Disease rating scale for bacterial blight incidence on walnut leaves and fruits (Aleta et al., 2001).

Rating Score	Description of Symptoms
0	No visible symptoms
1	Very small superficial spots confined to the inoculation point
2	Superficial spots less than 2 mm in diameter at the inoculation point
3	Blackening of the inoculation point measuring 2–5 mm in diameter, extending approximately 1 mm into inner tissues
4	Blackening around the inoculation point exceeding 5 mm in diameter, extending 2–4 mm into inner tissues

Sample collection and transportation

Diseased walnut plant specimens exhibiting characteristic bacterial blight symptoms were collected from leaves and fruits. To prevent contamination and bacterial deterioration, samples were placed in sterilized polyethylene bags, labeled with the collection location, date, and plant part, and transported to the Plant Pathology Laboratory, University of Poonch, Rawalakot, under cold conditions. Samples were processed within 24 h of collection (Schaad et al., 2001; Agrios, 2005).

Isolation and purification of the pathogen

The pathogen was isolated using standard bacteriological techniques. Symptomatic tissues were surface-sterilized with 70% ethanol and rinsed with sterile distilled water. Small portions of lesion edges, previously soaked in sterile distilled water, were streaked onto yeast extract-dextrose-calcium carbonate (YDC) and nutrient agar (NA) plates. Plates were incubated at 28 ± 2°C for 48-72 h. Individual colonies exhibiting typical *Xanthomonas*-like characteristics (yellow, mucoid, convex) were repeatedly subcultured to obtain pure cultures. For long-term preservation, purified isolates were stored in glycerol stocks at -80°C and on NA slants at 4°C (Lelliott and Stead, 1987; Schaad et al., 2001).

Pathogenicity testing (Koch's postulates)

Pathogenicity of selected bacterial isolates was evaluated under controlled conditions using healthy walnut plants or detached leaves. Bacterial suspensions were prepared in sterile distilled water at approximately 10^8 CFU/ml. Control plants were treated with sterile distilled water. Inoculation was performed via leaf infiltration or spraying. Inoculated plants were maintained at 25-28°C with high humidity (>85% relative humidity). Symptom development was monitored daily, and typical blight symptoms were recorded 7-14 days post-inoculation. Koch's postulates were confirmed by re-isolating the bacteria from infected tissues (Koch, 1884; Schaad et al., 2001; Belisario et al., 2002).

Morphological and biochemical characterization

Morphological characterization included colony appearance, color, and growth patterns on different media. Biochemical characterization was performed using standard tests, including Gram staining, oxidase, catalase, starch hydrolysis, gelatin liquefaction, and carbohydrate utilization. The results were consistent with the descriptions of *Xaj* (Lelliott and Stead, 1987; Schaad et al., 2001).

Statistical analysis

Data on disease incidence and severity were analyzed using descriptive statistics. Means and standard errors were calculated, and differences among locations were evaluated. Statistical analyses were performed using SPSS version 8.1 (Gomez and Gomez, 1984).

Results

Disease severity

The survey revealed that walnut bacterial blight was widespread across all surveyed districts of AJK, with notable variability in disease severity between locations and districts. The percent disease index (PDI), calculated using a standardized 0–4 disease rating scale, indicated moderate to extremely high disease severity in most districts.

In district Neelum, 10 sites were assessed, with PDI values ranging from 67.5% to 90%. Kutton and Athmuqam recorded the lowest severity (67.5%), whereas Jaggran and Batgran exhibited the highest, with PDI exceeding 90%. The district's mean PDI was 76.63%, indicating generally high disease pressure. Similarly, in district Jhelum, PDI ranged from 67.5% to 90%, with Ghaii Pura and Nokot showing the lowest severity and Ghasla the highest. The overall mean PDI in Jhelum was 69%, reflecting moderate disease incidence.

In district Muzaffarabad, PDI varied from 67.5% to 90%, with Kmhar Bandhi showing the highest severity and Nraat the lowest. The mean PDI of 76% suggests substantial disease pressure comparable to Neelum. District Bagh recorded comparatively lower PDI values (67.5-78.75%), with Junglari exhibiting the highest severity and Maloot and Sudhangali the lowest; the mean PDI was 71.25%, indicating moderate disease stress.

District Havelly showed slightly lower PDI scores, ranging from 67.5% to 78.75%. Khurshidabad had the highest severity (78.75%), while Mahmood Gali had the lowest (67.5%). The mean PDI of 74.75% suggests mild disease pressure. In district Poonch, PDI ranged from 67.5% to 78.75%, with Chotagala showing the highest severity and Rakkar, Gandali, and Hurnamehra the lowest. The mean PDI of 71.37% indicates moderate disease stress.

District Sudhnoti displayed PDI values between 67.5% and 90%, with Barral exhibiting the highest severity (90%) and Trarkhal, Papanaar, and Mang the lowest (67.5%). The mean PDI of 73.25% indicates mild to high disease pressure. Finally, district Kotli showed PDI values from 67.5% to 78.75%, with Miti and Matharani recording the highest severity (78.75%) and Jandrote, Dabsi, Banala, and Majhan the lowest (67.5%). Kotli's mean PDI of 71.25% reflects moderate disease stress. Detailed PDI values for each surveyed location are presented in Figures 1-8.

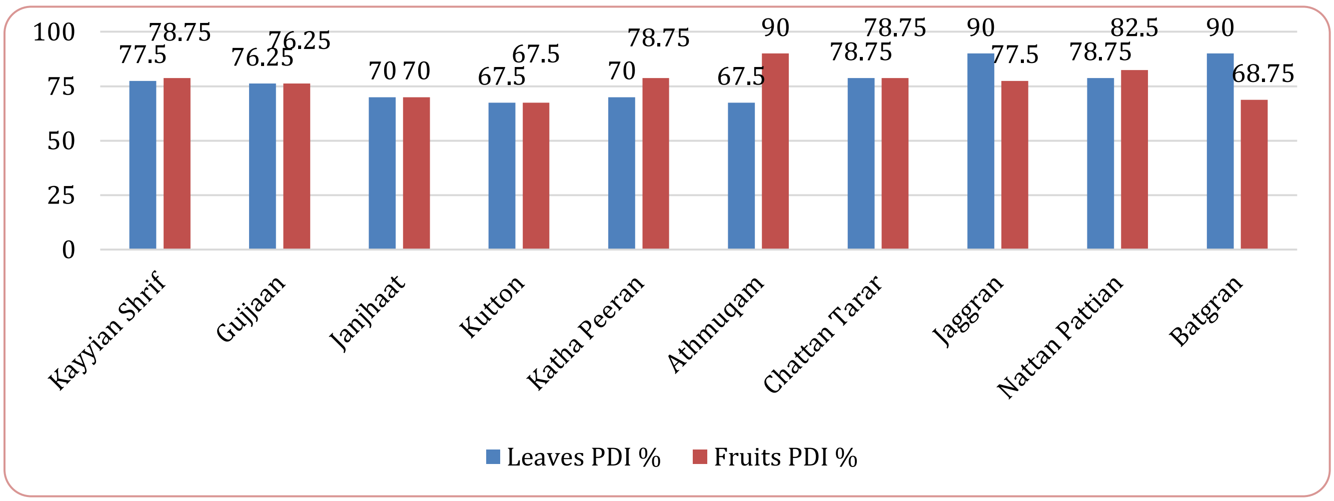

Figure 1. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Neelum, AJK, Pakistan.

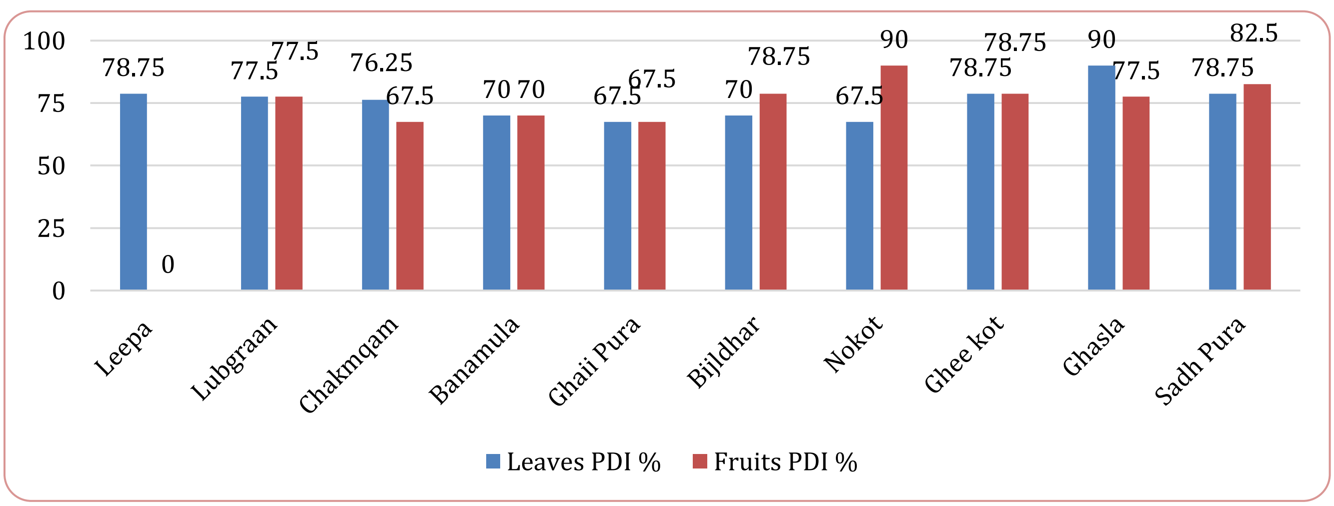

Figure 2. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Jhelum, AJK, Pakistan.

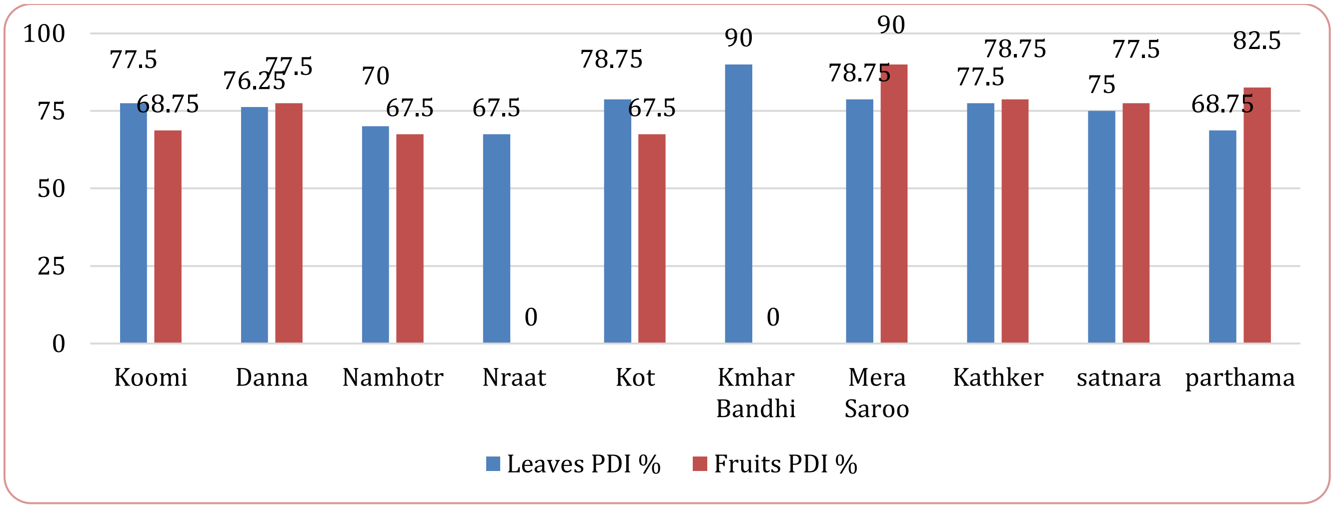

Figure 3. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Muzaffrabad, AJK, Pakistan.

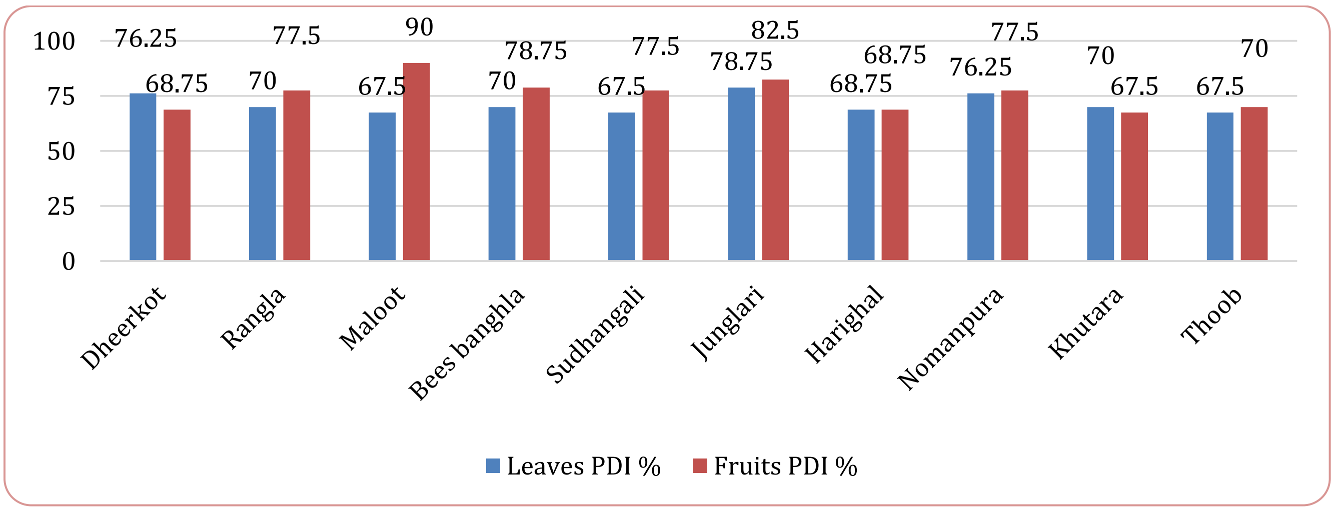

Figure 4. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Bagh, AJK, Pakistan.

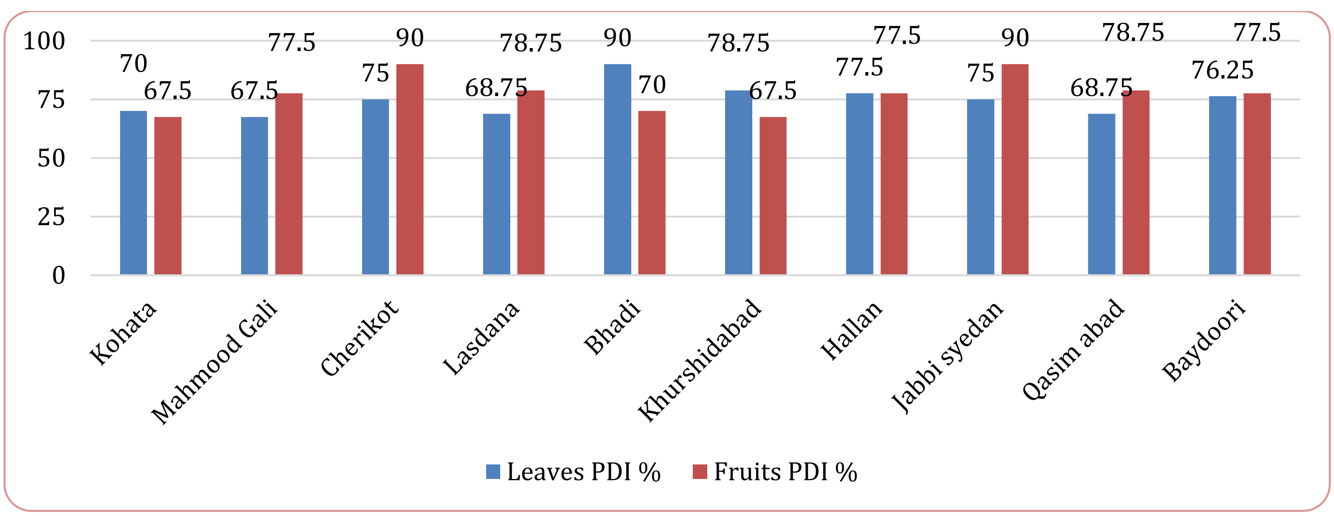

Figure 5. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Havelly, AJK, Pakistan.

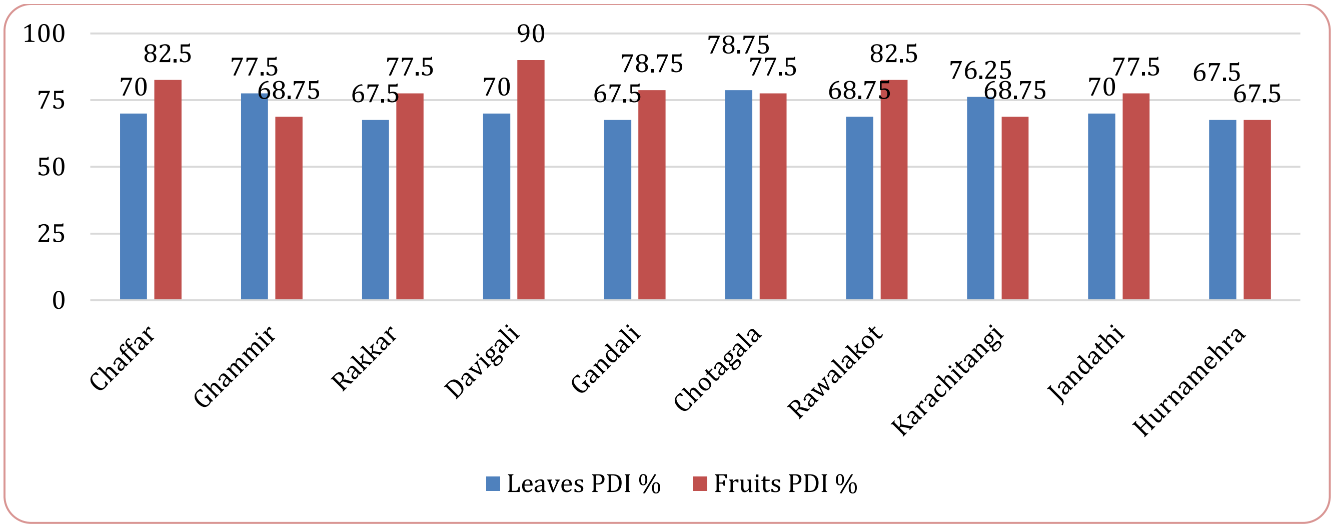

Figure 6. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Poonch, AJK, Pakistan.

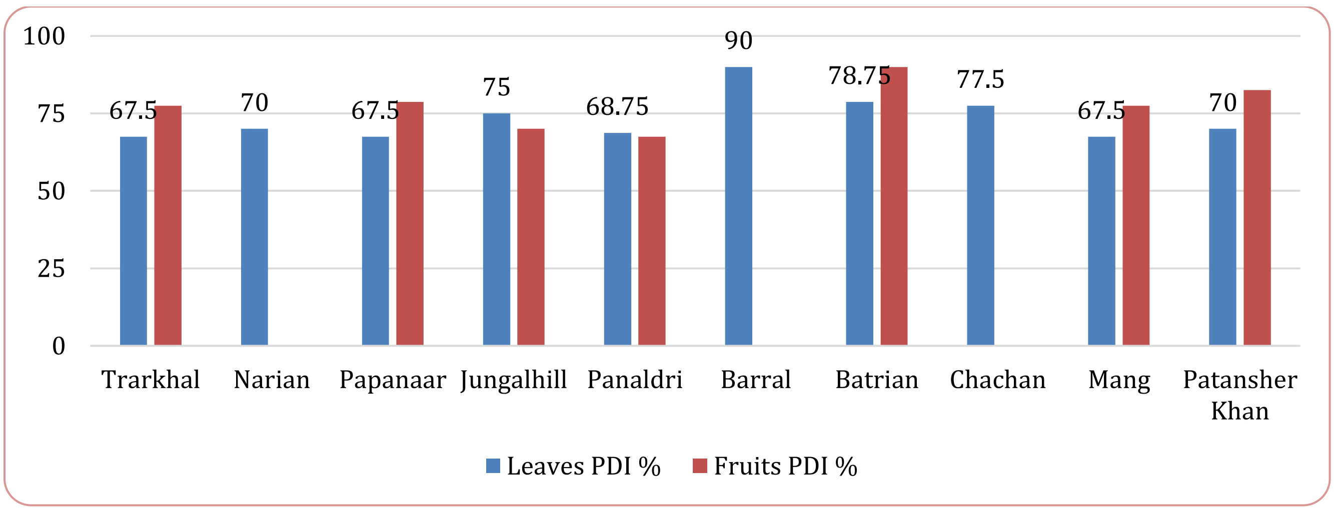

Figure 7. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Sudhnoti, AJK, Pakistan.

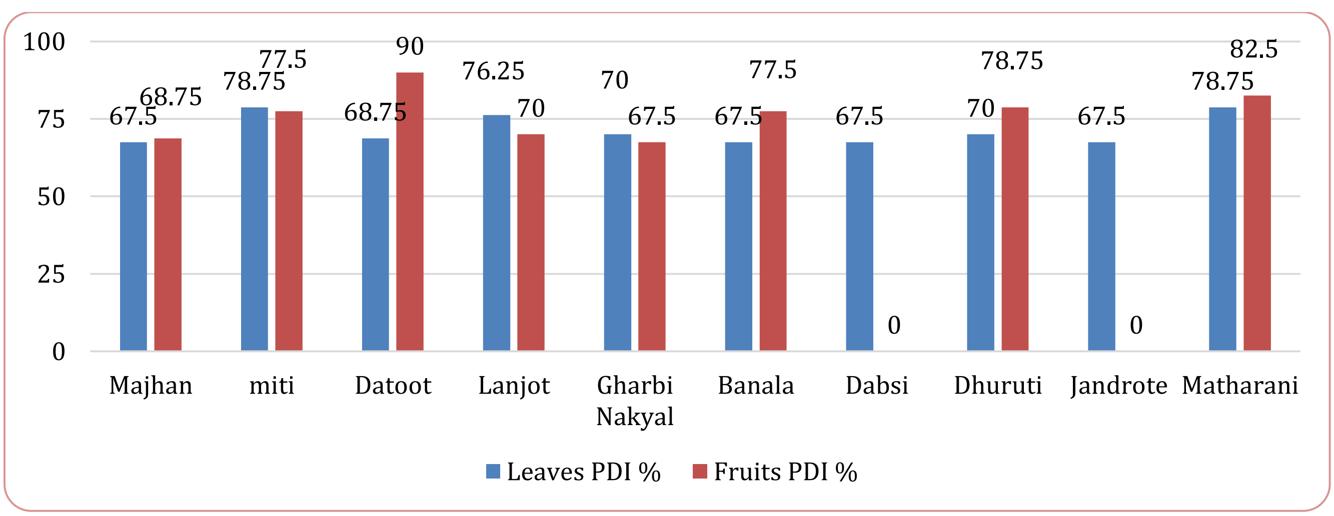

Figure 8. Location-specific % DI of walnut bacterial blight on leaves and fruits in district Kotli, AJK, Pakistan.

Disease incidence and prevalence

The incidence of walnut bacterial blight in district Neelum, AJK, ranged from 70% to 100% across the ten surveyed locations, with the highest incidence recorded at Janjhaat (100%). The disease was observed in all surveyed sites, resulting in 100% disease prevalence for the district. In district Jhelum, disease incidence ranged from 0% to 90%, with Bijldhar and Ghee Kot exhibiting the highest incidence (90%), leading to an overall disease prevalence of 80%. In district Muzaffarabad, incidence ranged from 0% to 80%, with the highest values observed at Koomi, Namhotr, Kathker, and Parthama (80%), resulting in a 70% prevalence. District Bagh showed disease incidence between 40% and 90%, with Dheerkot, Bees Banghla, and Sudhangali recording the highest incidence (90%), corresponding to 90% disease prevalence. In district Haveli, incidence varied from 50% to 90%, with the highest incidence at Jabbi Syedan (90%), resulting in 100% prevalence. District Poonch exhibited incidence values from 20% to 90%, with Chaffar and Chotagala recording the highest incidence (90%), yielding a prevalence of 70%. In district Sudhnoti, incidence ranged from 0% to 80%, with Trarkhal, Papanaar, Panaldri, and Patansher Khan showing the highest values (80%), leading to a 60% prevalence. Finally, in district Kotli, incidence ranged from 0% to 90%, with the highest incidence at Majhan (90%), resulting in 60% prevalence. Figures 9-16 present detailed disease incidence across all surveyed locations, while Figure 17 illustrates the overall disease prevalence in each district.

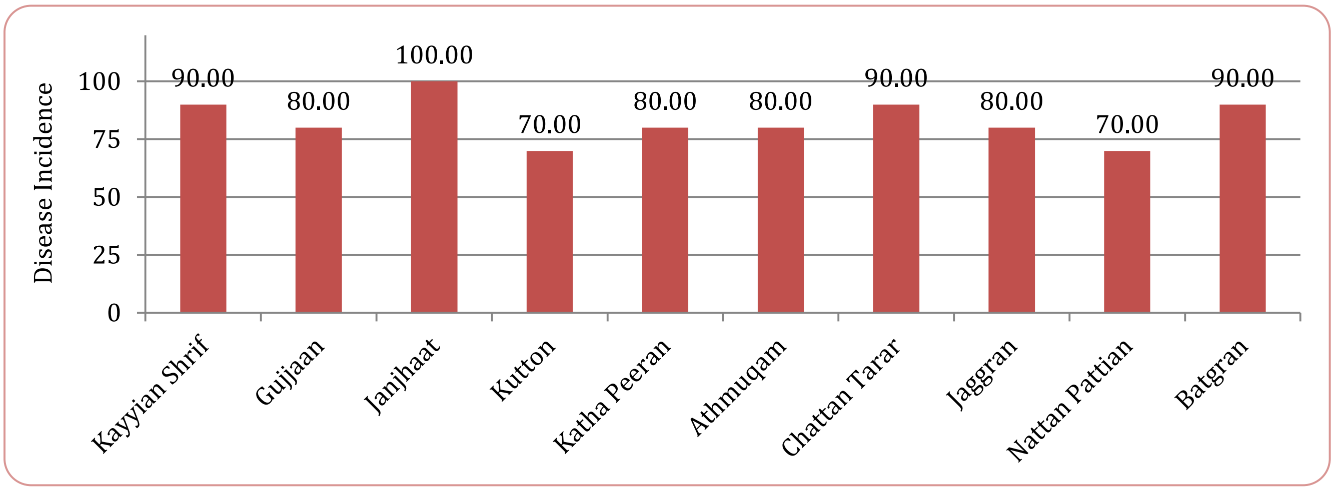

Figure 9. Disease incidence (%) of walnut bacterial blight across different locations in district Neelum.

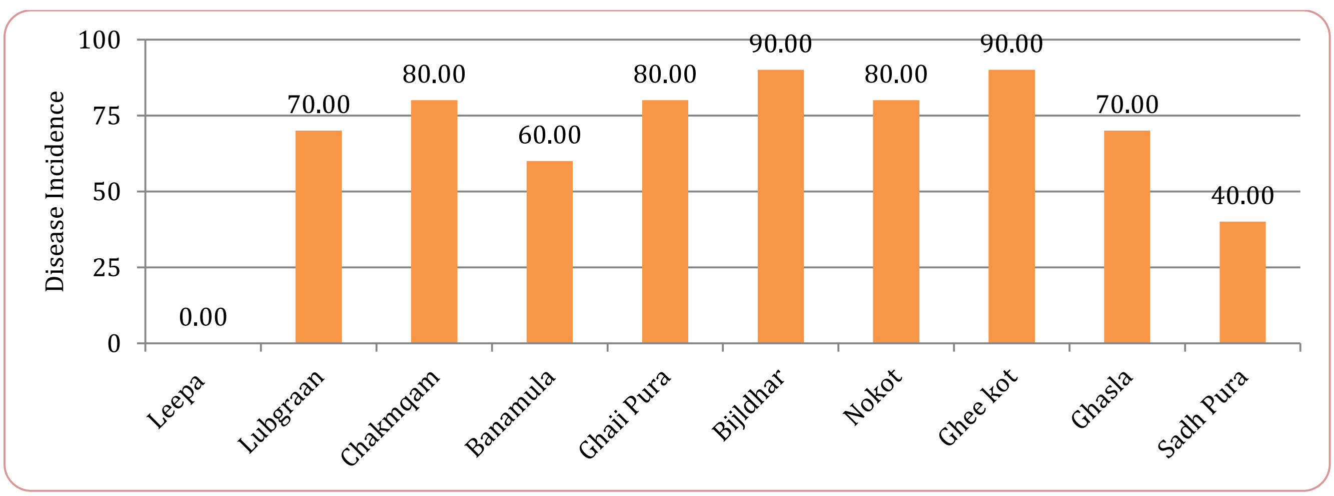

Figure 10. Disease incidence (%) of walnut bacterial blight across different locations in district Jhelum.

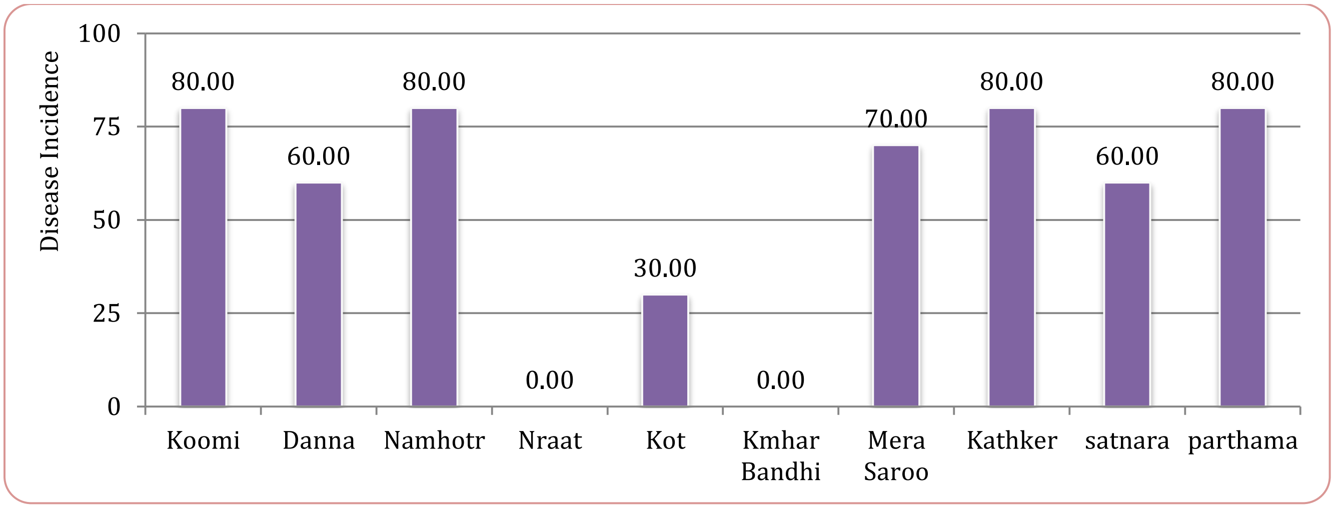

Figure 11. Disease incidence (%) of walnut bacterial blight across different locations in district Muzaffrabad.

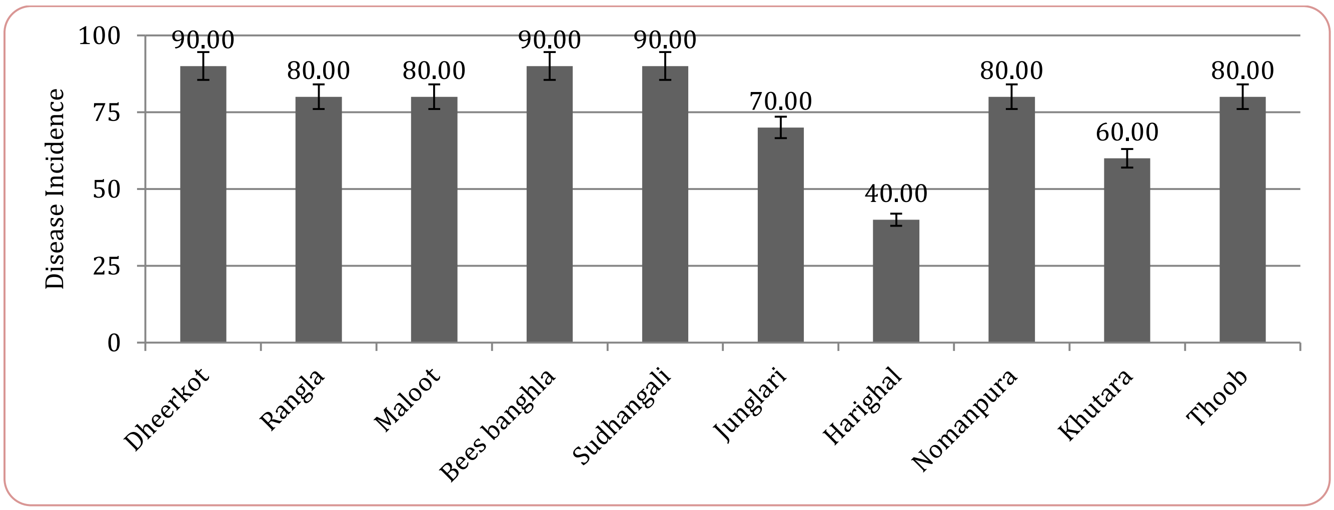

Figure 12. Disease incidence (%) of walnut bacterial blight across different locations in district Bagh.

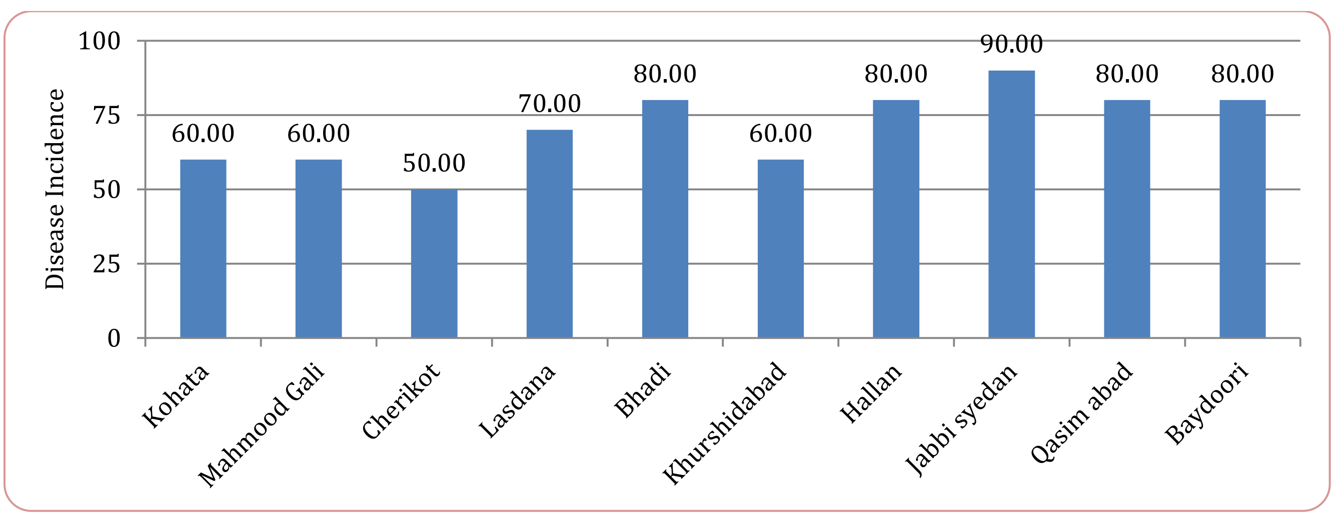

Figure 13. Disease incidence (%) of walnut bacterial blight across different locations in district Havelly.

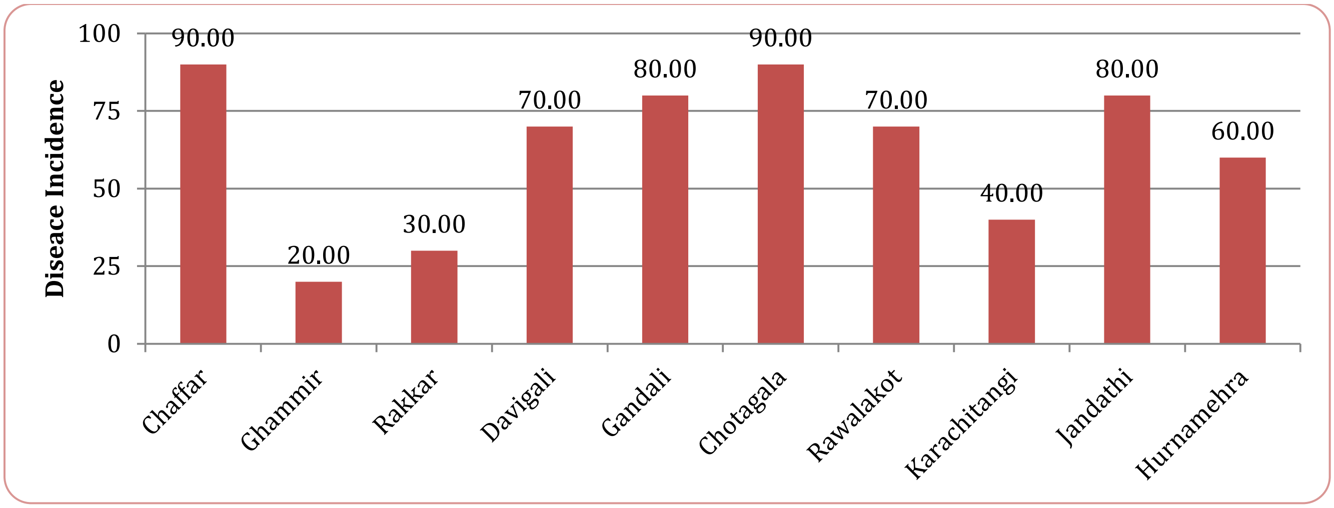

Figure 14. Disease incidence (%) of walnut bacterial blight across different locations in district Poonch.

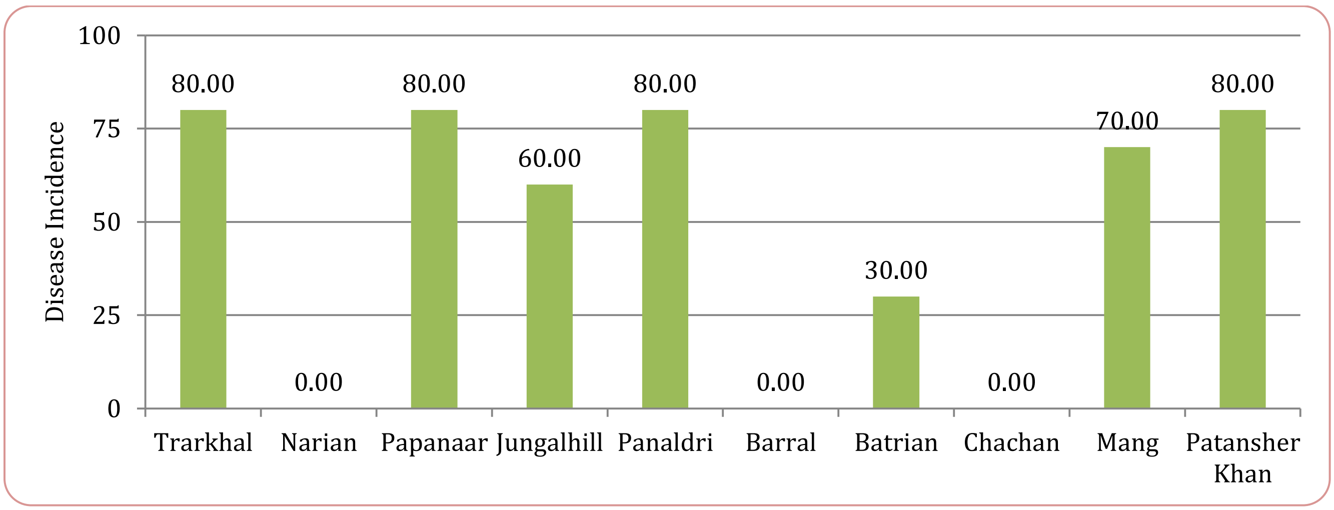

Figure 15. Disease incidence (%) of walnut bacterial blight across different locations in district Sudhnoti.

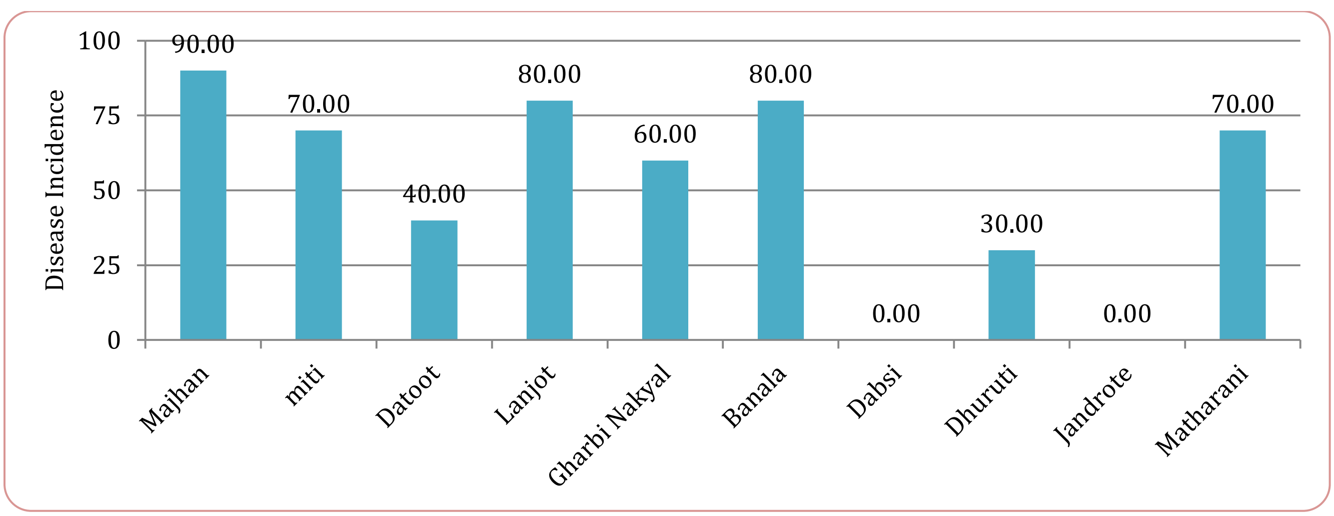

Figure 16. Disease incidence (%) of walnut bacterial blight across different locations in district Kotli.

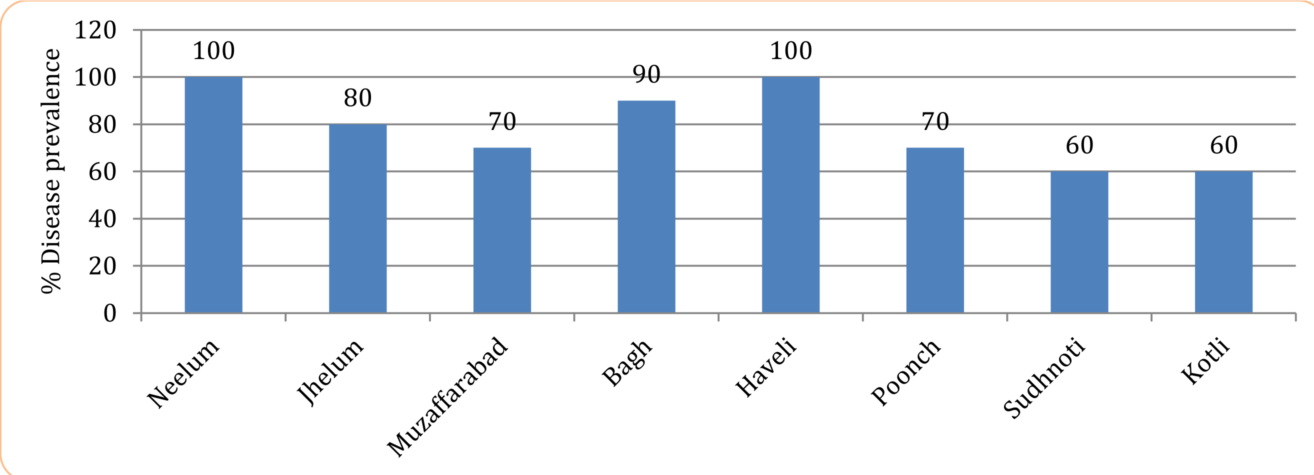

Figure 17. Disease prevalence (%) of walnut bacterial blight across eight districts of AJK, Pakistan.

Pathogen isolation and cultural characteristics

Bacterial isolates were consistently recovered from symptomatic walnut leaves and fruits collected from eight districts of AJK, Pakistan. Of the 513 samples processed, 80% yielded bacterial colonies exhibiting uniform morphological characteristics. On nutrient agar, colonies appeared within 48-72 h of incubation at 28 ± 2°C, forming yellow, mucoid, smooth, circular, and convex colonies with entire margins. Colony diameters ranged from 2.0 to 4.0 mm after 72 h. Similar colony morphology was observed on yeast extract dextrose calcium carbonate (YDC) medium, with more pronounced yellow pigmentation.

Biochemical characterization

The pathogenic isolates exhibited consistent biochemical profiles, including Gram-negative reaction, positive catalase activity, negative oxidase reaction, starch hydrolysis positive, gelatin liquefaction positive, and acid production from glucose, while being negative for indole production. The results of additional morphological and biochemical tests are summarized in Table 2.

Table 2. Morphological and biochemical characteristics used for the identification of *X. arboricola* pv. *juglandis*.

Test Category	Test Name	Reaction	Reference
Morphological	Colony color on NA	Yellow	Schaad et al. (2001)
	Colony texture	Mucoid	Lelliott and Stead (1987)
	Colony shape	Circular, convex	Schaad et al. (2001)
Staining	Gram reaction	Negative	Lelliott and Stead (1987)
Enzymatic	Catalase	Positive	Schaad et al. (2001)
	Oxidase	Negative	Schaad et al. (2001)
	Urease	Negative	Lelliott and Stead (1987)
	Gelatin liquefaction	Positive	Schaad et al. (2001)
Hydrolytic	Starch hydrolysis	Positive	Lelliott and Stead (1987)
	Tween-80 hydrolysis	Positive	Schaad et al. (2001)
Physiological	Growth at 37 °C	Negative	Schaad et al. (2001)
	Growth in 5% NaCl	Negative	Lelliott and Stead (1987)
Carbon Utilization	Glucose	Positive	Schaad et al. (2001)
	Sucrose	Positive	Lelliott and Stead (1987)
	Lactose	Negative	Schaad et al. (2001)
Other Tests	H_2S production	Negative	Schaad et al. (2001)
	Indole production	Negative	Lelliott and Stead (1987)

Pathogenicity tests

The pathogenicity of selected bacterial isolates was assessed under controlled conditions on young walnut fruits and healthy leaves. Water-soaked lesions first appeared five to seven days post-inoculation. Over time, the lesions enlarged, darkened from brown to black, and closely resembled the symptoms observed in the field. Control plants inoculated with sterile distilled water remained symptom-free. Disease severity on inoculated fruits ranged from 2 to 4 on a 0–4 scale. Koch's postulates were fulfilled, as the pathogen was successfully re-isolated from artificially infected tissues, exhibiting colony morphology identical to the original isolates. The high recovery rate of consistent bacterial isolates from symptomatic tissues, coupled with confirmed pathogenicity, establishes *X. arboricola* pv. *juglandis* as the primary causal agent of walnut blight in Azad Jammu and Kashmir. These findings provide a strong foundation for further biochemical and molecular characterization and offer essential baseline data for developing effective disease management strategies.

Discussion

The present study provides the first comprehensive account of the surveillance, severity assessment, and pathogen characterization of walnut bacterial blight in AJK, Pakistan. Integrating extensive field surveys with pathogenicity testing and molecular identification confirmed *X. arboricola* pv. *juglandis* as the sole causal agent and highlighted its widespread distribution and high destructive potential in the region. High percent disease index (PDI) values recorded across all surveyed districts indicate that bacterial blight is a major constraint to walnut production in AJK. Mean PDI values exceeding 70% in Neelum, Jhelum, Muzaffarabad, Bagh, Haveli, Poonch, and Sudhnoti districts reflect severe disease pressure and favorable environmental conditions for pathogen proliferation. Comparable disease severity has been reported in walnut-growing regions of Europe and Asia, where extended spring rainfall and moderate temperatures promote epiphytic multiplication and subsequent infection of flowers and young fruits (Miller, 1946; Lamichhane, 2014).

Observed spatial variation in disease severity among districts and locations may be attributed to differences in microclimate, orchard management practices, and host susceptibility. Districts with higher rainfall and prolonged leaf wetness exhibited greater disease intensity, supporting earlier findings that high environmental moisture is a critical driver of walnut blight epidemics (Teviotdale, 2002). Conversely, comparatively lower PDI values in certain locations of Muzaffarabad and Jhelum districts likely reflect less favorable microclimates or reduced inoculum pressure. High disease incidence and prevalence across surveyed areas further confirm that walnut bacterial blight is well established in AJK, consistent with reports from other walnut-producing regions where the pathogen persists epiphytically on buds and twigs and initiates annual infection cycles (Moragrega and Llorente, 2023).

Consistent isolation of yellow, mucoid bacterial colonies from symptomatic tissues across all districts strongly supports the association of *Xanthomonas* spp. with walnut blight in AJK. Colony morphology on nutrient agar and YDC medium corresponded with previous descriptions of *X. arboricola* pv. *juglandis* (Bradbury, 1986; Schaad et al., 2001). Production of yellow xanthomonadin pigmentation enhances bacterial survival under field conditions by protecting against photooxidative damage (Assis et al., 2021). Uniform cultural characteristics among isolates suggest a genetically conserved pathogen population, a pattern also reported in Europe and Asia (Lamichhane, 2014).

Artificial inoculation reproduced typical walnut blight symptoms on leaves and immature fruits, confirming the pathogenicity of the isolates. Symptom development, beginning as water-soaked lesions and progressing to necrotic black spots, mirrored natural field infections. Re-isolation of the pathogen and the absence of symptoms in control plants fulfilled Koch's postulates, establishing a definitive causal relationship. The rapid symptom expression observed highlights the pathogen's aggressive nature and explains the high disease severity documented in field surveys. Collectively, these findings indicate that walnut bacterial blight is a serious and persistent threat to walnut production in AJK. The pathogen's ability to survive epiphytically, combined with favorable climatic conditions, creates ideal conditions for recurring epidemics, emphasizing the urgent need for integrated disease management strategies (Teviotdale, 2002; Lamichhane, 2014).

This study provides a critical baseline for the development of effective management approaches, including the selection of resistant cultivars, optimization of cultural practices, and judicious use of bactericides. Molecular characterization lays the groundwork for future studies on pathogen diversity, population structure, and epidemiology in Pakistan and neighboring regions.

Conclusion

This study represents the first comprehensive evaluation of walnut bacterial blight in Azad Jammu and Kashmir, Pakistan. With moderate to extremely high disease severity on walnut leaves and fruits, the disease is widely distributed throughout surveyed areas. Walnut bacterial blight poses a significant and persistent threat to walnut production in the temperate zones of AJK, as evidenced by high PDI values and increasing disease incidence and prevalence. Spatial variation in disease intensity among districts underscores the influence of host susceptibility, orchard management practices, and local environmental conditions on disease development.

Authors' Contributions

BM and RA conceived and designed the study. BM, RA, and MT conducted field surveys and collected diseased samples. BM and UM carried out the isolation and identification of the pathogen, performed the statistical and data analyses, and prepared the original draft of the manuscript. MAI and MT provided critical scientific guidance, constructive feedback, and valuable suggestions to enhance the quality, coherence, and clarity of the manuscript. RA supervised the overall research activities and ensured adherence to scientific rigor. All authors critically reviewed and approved the final version of the manuscript and agreed to be accountable for all aspects of the work, ensuring accuracy, transparency, and integrity in the reporting of results.

Research Funding

This research did not receive any grant from funding agencies.

Conflict of Interest

The authors declare no conflict of interest.

Sustainable Development Goals Targeted

SDG 2: Zero Hunger
SDG 12: Responsible Consumption and Production
SDG 15: Life on Land

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