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PREVALENCE AND CHARACTERISTICS OF CHERRY SHOT-HOLE DISEASE IN GILGIT-BALTISTAN

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ABSTRACT

Shot-hole disease is one of the most common and significant diseases affecting cherry trees in Gilgit-Baltistan, Pakistan, every year. It results in small reddish areas on leaves and fruits, forming characteristic holes. However, the pathogen associated with the disease, its pathogenicity, and its growth performance under different environmental conditions remains unknown. In this study, the pathogen associated with shot-hole disease was identified as *W. carpophilus*. Furthermore, variations in pathogenicity were observed among the five isolates of the pathogen (SRA-1, SRA-2, SRA-3, SRA-4, and SRA-5), with SRA-5 exhibiting smaller lesion sizes and SRA-1 to SRA-4 displaying larger lesion sizes. Differential responses to temperature variations were noted, with isolates SRA-1 and SRA-4 growing faster at different temperature regimes. Conidia count and biomass measurements revealed isolate-specific patterns under varying temperature regimes. Growth performance and colony characteristics varied among isolates. Environmental responses indicated sensitivity to osmotic potential and pH, as well as preferences for different culture media. This research provides crucial insights into the morphology, behavior, and adaptation of *W. carpophilus*. These findings will facilitate the establishment of management strategies against shot-hole disease in cherry orchards.

Keywords: Cherry, Shot-hole disease, pathogenicity, morphological characteristics, environmental responses.

INTRODUCTION

Sweet cherry (*Prunus avium* L.) cultivation in Pakistan in the valleys and foothills of Gilgit-Baltistan *viz.,* Gilgit, Hunza, and Nagar districts are major cherry-producing areas while in Baluchistan includes Quetta, Kalat, Pishin, Loralai, Mastung, Zhob, and Swat. The total cherry production is approximately 400 hectares in Pakistan, producing 1660 tons of cherry fruit, annually, whereas the production of cherry in GB was 2384/1302 hectares (FAOSTAT, 2019; Noor *et al*., 2020; Hassan *et al*., 2022). GB holds the country's largest share (64.77%) of cherry production. Given GB's proximity to China's Xinjiang province, Pakistan sees an opportunity to leverage the China-Pakistan Economic Corridor (CPEC) for mutual economic benefits (Spies, 2021). Consequently, Pakistan has formally requested China to facilitate its exports, including agricultural products like cherry, into the Chinese market. However, fungal shot hole disease is considered a significant quarantine disease of cherry under phytosanitary laws and regulations of both countries (Teviotdale *et al.*, 1997; CPLR, 2023). It is one of the serious diseases of the *Prunus* species in many parts of the temperate to semiarid regions of the world including GB. This disease, named after the small holes, it creates on sweet cherry tree leaves during spring, also affects fruits, branches, buds, and flower calyxes. The shot-hole disease manifests as a visual tapestry on leaves, where a spectrum of symptoms unfolds. This spectrum spans from diminutive reddish or purplish spots adorned with yellow halos, gradually evolving into larger, irregular

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reddish-brown spots along the leaf margins. As the process advances, these afflicted spots exhibit an intriguing behavior – the center gradually drops out, creating a phenomenon reminiscent of a "shot-hole." This intriguing progression paints a dynamic portrait of the disease's lifecycle. However, shot-hole disease does not limit its canvas to leaves alone; fruits and twigs also provide a substrate for its artistic endeavors. On twigs and fruits, the symptoms emerge as discrete, small black spots, a prelude to a transformation as they expand and deepen, giving way to sunken areas. The causal agent of shot hole disease is a fungal species known as *Wilsonomyces carpophilus* (Lev.) Adask., This pathogen, formerly named as *Helminthosporium carpophilum,* has undergone a number of name changes and taxonomic affiliations, as well as being recognized by many synonyms including *Stigmina carpophila*, *Coryneum beijerinkii*, *Clasterosporium carpophilum*, *Thyrostroma carpophilum*, *Sciniatosporium carpophilum*, and *Sporocadus carpophilus* (Teviotdale *et al.*, 1997; Sundaravadivelan *et al.*, 2014; Dar *et al.*, 2017; Ye *et al.*, 2020). The complex journey of this fungal species through multiple names reflects the evolving understanding of its morphology, genetic relationships, and ecological roles. It is noteworthy that *Wilsonomyces carpophilus* is recognized as an anamorphic (asexual) stage of the broader genus *Wilsonomyces*, a classification that has implications for its taxonomic placement and evolutionary history. This genus, in turn, finds its home within the Dothideomycetes class, Pleosporomycetidae subclass, and Pleosporales order, specifically within the family Dothidotthiaceae (Ellis, 1959; Han *et al.*, 2022). Shot hole disease in cherry trees of GB is influenced by various environmental factors, including air temperature, rainfall, relative humidity, and duration of leaf wetness. These factors contribute to the susceptibility of cherry trees to the disease. Consequently, shot hole disease poses a continuous threat to the cherry tree population in GB, resulting in substantial losses (Verma, 2005; Nabi *et al.*, 2018). The risk associated with shot hole disease in cherry trees arises from multiple factors. One factor is the local survival of inoculum during winter or summer, which can persist in twig cankers, leaves, buds, weeds, and plant debris. Additionally, the pathogen population comprises a diverse range of virulent strains, further increasing the risk. The limited understanding of the etiology of the plant pathogen responsible for shot hole disease adds to the challenges (Muhammad *et al.*, 2022; Abbas *et al.*, 2023). As this is a quarantine disease, it is crucial to understand the etiology and growth performance of shot hole disease to meet the phytosanitary requirements and facilitate cherry exports. Without such knowledge, effective management strategies cannot be developed. The objectives of this research are multifaceted and essential for addressing the challenges posed by cherry shot-hole disease effectively. Firstly, a comprehensive survey will be conducted to gain insights into the prevalence, distribution, and pathogenicity of this disease. This aspect of the research will involve extensive data collection and analysis to better understand the geographic scope and severity of the issue. Secondly, the research aims to isolate and characterize the fungal isolates responsible for shot-hole disease. This involves a detailed assessment of morphological features and phenotypic traits, which are crucial for identifying and classifying the pathogens accurately. By characterizing these isolates, the research seeks to provide a foundation for subsequent studies on their behavior and interactions with cherry trees. Lastly, the impact of various environmental factors on the growth of these fungal isolates will be investigated. This includes analyzing the influence of pH levels, culture media, osmotic potentials, and temperature regimes on their growth patterns. Understanding how these factors affect the pathogen's development is vital for devising targeted management strategies that can mitigate the impact of cherry shot-hole disease. Overall, these research objectives are interlinked and pivotal for advancing our knowledge of this disease and developing effective means of control.

MATERIALS AND METHODS

Survey and sampling: A thorough survey was undertaken to investigate the prevalence of cherry shot-hole disease in District Gilgit, spanning the spring and summer periods from 2022 to 2023. This comprehensive study involved systematic efforts, including the examination of multiple sample plots, each chosen randomly. Five cherry orchards were selected at random. Within each orchard, five cherry trees were chosen in a random manner. Subsequently, a total of 30 leaves and 10 fruits were methodically collected from each selected tree (Ye *et al.*, 2020). The fruits and leaves were selected based on visual symptoms, targeting instances of discoloration, punctures, brown spots, holes, and necrosis.

Isolation: Cherry leaves and fruit samples were collected and transported to the Plant Pathology Laboratory at the Institute of Plant and Disease Management (IPDM) in Gilgit. To ensure the establishment of an accessible repository of infected specimens, dry samples of afflicted

leaves and fruits were systematically deposited in the herbarium of IPDM Gilgit. For the isolation of the causal agent responsible for the observed diseases, sections of the margins of infected lesions on both fruits and leaves were delicately excised into small pieces measuring 5 x 5 mm. Following this, the surface of these tissue fragments was subjected to disinfection by immersing them in 75% ethanol for 1 minute, followed by subsequent treatment with 3% sodium hypochlorite (NaOCl) for a duration of 3 minutes. The disinfected samples underwent three rinses with sterile distilled water and were then air-dried within sterilized Petri dishes. Each symptomatic fruit and leaf sample contributed three to five pieces, which were individually placed into 90 mm Petri dishes containing full-strength Potato Dextrose Agar (PDA) medium with a concentration of 39 g/L. These Petri dishes were incubated at a temperature of 24°C under a 12-hour photoperiod within a light incubator over a period of 5 days. Mycelial fragments obtained from the growing colony margins were subsequently transferred onto fresh PDA medium for further propagation. To single-conidia isolation, conidia were meticulously removed from the colonies using a sterile needle and then suspended in 1 mL of sterile distilled water containing 0.1% Tween 20. A 50 mL aliquot of the conidial suspension was evenly spread over water agar within a Petri dish. Following an incubation period of 24 hours at 24°C, germinated conidia were identified and isolated under a stereomicroscope. These single-germinated conidia were then transferred to PDA plates and cultivated for an additional 36 hours at 24°C to encourage mycelium development. Resulting colonies originating from single conidia were subsequently cultured on fresh PDA medium, and for long-term preservation, pure cultures were stored in a solution of 15% glycerol at a temperature of -20°C.

Identification: Following a series of purification procedures, pure fungal cultures were successfully acquired for experimentation. To ascertain controlled mycelial growth rates, a deliberate effort was made to regulate conditions across three distinct growth media as aforementioned. Each growth medium was evenly distributed across 10 Petri dishes, and the experiment was carried out over a period of five days. The mycelium's progression was closely monitored throughout the experiment. Daily assessments were conducted, involving the measurement of mycelium diameters along two perpendicular axes. This comprehensive approach provided accurate insights into the mycelial growth dynamics. To thoroughly scrutinize the impact of the three different growth media on the fungal isolates' growth, gathered data for each individual day underwent rigorous analysis of variance. After the variance analysis, a multiple-range test was executed to facilitate the comparison of average growth levels among the diverse growth media. This enabled a nuanced understanding of how each medium influenced the growth of the fungal isolates. For comprehensive statistical analysis, the Statistics software package was utilized, ensuring robust and reliable results. As part of the identification process for fungal colonies, a hybrid approach incorporating various identification keys was adopted. These keys, authored by experts like Adaskaveg *et al*. (1990) and Ellis and Ellis (1985) and (Ellis and Ellis, 1985) documented both microscopic and macroscopic symptoms (Adaskaveg *et al.*, 1990). By harnessing the insights provided by these references, a comprehensive and multifaceted strategy was implemented to accurately identify the fungal colonies under examination (Lücking *et al*., 2020).

Pathogenicity Tests: The pathogenicity experiments were carried out over a two-month period, spanning from June to July 2023, within the Plant Pathology laboratory of the Institute of Plant and Disease Management (IPDM) in Gilgit. The primary objective of this experiment was to test Koch's postulates using five distinct isolates of shot hole fungus with the utilization of detached leaves. In pursuit of this objective, young leaves were collected from healthy sweet cherry trees to serve as the experimental substrate. Inoculation procedures involved the utilization of mycelial plugs measuring 6 mm in diameter. Prior to inoculation, the fungal isolates underwent individual cultivation on PDA medium for a duration of 5 days, maintaining a constant temperature of 24°C under a 12-hour photoperiod. The experimental conditions were upheld in an aseptic environment to ensure the validity of the results. To create a controlled environment for inoculation, comparable healthy leaves were chosen, subjected to thorough washing with tap water, followed by a 3-minute surface sterilization using a 1% sodium hypochlorite (NaOCl) solution. Subsequently, the plant materials were rinsed three times with sterile distilled water and air-dried on paper towels within a laminar flow cabinet for approximately one hours. In the inoculation process, a mycelial plug with a diameter of 6 mm was placed on leaves, with the mycelium facing downward. Control groups underwent a mock inoculation using non-colonized PDA plugs. The inoculated leaves were placed in trays lined

with tissue paper moistened with water, and these trays were covered with a plastic lid to maintain high humidity levels. The trays were placed on a laboratory bench at room temperature until the manifestation of symptoms. Each pathogenicity test was executed with three replicates, and aseptic procedures were followed throughout the process. Following the manifestation of symptoms, the fungus was isolated anew from the infected leaves using the method. Fungi that were isolated in accordance with Koch's postulates were subsequently transferred to fresh plates and allowed to cultivate for a duration of 5 to 7 days, awaiting macroscopic and microscopic identification. Quantitative measurements of lesion size were carried out by determining the length and width of the lesions using a ruler. Subsequently, the oval area of the lesions was calculated based on these measurements, providing an objective indicator of the lesion size.

Growth performance at different growth media: For the purpose of morphological identification, five isolates representing diverse morphological groups were selected. Among these, three were derived from leaves and two from fruits. The morphological evaluation of these isolates was carried out following their growth on three distinct growth media: Malt Extract Agar (MEA), Czapek Yeast Extract Agar (CYA), and Potato Dextrose Agar (PDA). These growth media can be prepared as; MEA is prepared by combining malt extract, glucose, peptone, and agar in specific proportions. The composition per liter of MEA consists of 20 grams of malt extract, 20 grams of glucose, 20 grams of peptone, and 20 grams of agar. Potato Dextrose Broth (PDB) is formulated using potato infusion and dextrose. To create PDB, 4 grams of potato infusion and 20 grams of dextrose are used per liter of the growth medium. CYA is another growth medium designed with specific constituents. The composition per liter of CYA includes 3 grams of $NaNO_3$, 1 gram of KH_2PO_4, 0.5 grams of $MgSO_4.7H_2O$, 0.5 grams of KCl, 0.01 grams of $FeSO_4.7H_2O$, 30 grams of glucose, and 20 grams of agar. Finally, PDA is composed of potatoes, glucose, and agar. For each liter of PDA, 200 grams of potatoes, 20 grams of glucose, and 20 grams of agar are used. Each medium was carefully distributed into Petri dishes measuring approximately 9 cm in diameter, each containing 15 mL of the designated medium. Subsequently, a 6 mm mycelial disk obtained from 7-day-old isolates was inoculated at the center of these dishes. After a cultivation period of 5 days at 20, 24 and 28°C, the colony diameter (cm) was measured, with three replicates maintained for each isolate, ensuring robust data. The anamorph characteristics of the isolates were scrutinized during these assessments. This encompassed the examination of conidiophores and conidia, encompassing their size and shape. To facilitate conidia observation, the isolates were placed in water on a glass slide and observed using an Olympus compound microscope, and photographs were taken (Adaskaveg *et al.*, 1990; Alaniz *et al.*, 2007).

Colony diameter, biomass, and conidial production: To comprehensively characterize the shot hole fungal isolates, several parameters including colony diameter (cm), color, shape, texture, margin characteristics biomass (g and/or mg), and conidia production were meticulously recorded on Potato Dextrose Agar (PDA) medium. The PDA medium was formulated through a process involving the cooking of 200 g of peeled potatoes in 1000 mL of distilled water for 10 minutes. The resulting potato infusion was filtered through four layers of cheesecloth, after which 20 g of agar and 20 g of dextrose were added. Following formulation, PDA was autoclaved at 121°C for 20 and 30 minutes, respectively. Initially, PDA was employed to assess the sporulation capabilities of the shot hole fungal isolates. For the evaluation of colony diameter, biomass, and conidia production, three distinct temperature regimes (20, 24, and 28°C) were employed. In this process, Petri dishes measuring 9 cm in diameter were utilized. Each dish contained 15 mL of PDA, into which a 6 mm mycelial disk from each isolate was introduced. The colony diameter (cm) was measured after a 5-day incubation period, with three replicate plates maintained for each isolate. To ascertain biomass and conidia production, a separate approach was undertaken. A 6 mm mycelial disk of each isolate was positioned at the center of a Petri dish lined with sterilized cellophane. These dishes were maintained for a 14-day period. Afterward, the conidia were carefully harvested from the petri dishes using a sterilized spatula, with 10 mL of sterilized distilled water (SDW) employed to facilitate this process. The conidia containing SDW suspension, along with mycelia and PDA debris, underwent filtration through two layers of sterilized cheesecloth to eliminate mycelia and debris. The resulting conidia suspensions were quantified using a hemocytometer. Meanwhile, for biomass measurement, mycelial disks of approximately 6 mm were placed at the center of petri dishes that were overlaid with sterile cellophane. The mycelia were then carefully extracted from the cellophane, dried in an oven at 60°C for 48 hours, and subsequently weighed to determine fungal biomass

(Dar *et al.*, 2017).

Growth performance at different pH and Osmotic potentials: To further explore the phenotypic characteristics of the isolates, a comprehensive set of experiments was conducted involving varied growth media, pH levels, and osmotic potentials. The exploration extended to the assessment of growth under varying osmotic potentials. This was achieved by adjusting PDA with different osmotic potentials using KCl. Concentrations ranged from 0.1 MPa (control) to 5.0 MPa, with KCl concentrations ranging from 0 to 5 g/L. A 6 mm mycelial plug from 7-day-old isolates was introduced at the center of the PDA medium and subjected to incubation at 28°C for 5 days. Post-incubation, the colony diameter (cm) was carefully measured to quantify the growth under these diverse osmotic potential conditions. Additionally, the isolates' growth response was evaluated across a spectrum of pH conditions, encompassing pH values of 3, 4, 5, 6, 7, and 8. To establish different pH levels, pH values of 3, 4, 5, and 6 were adjusted using a 0.1 M citric acid sodium phosphate buffer, while pH values of 7 and 8 were achieved using 0.05 M Tris-HCL Buffer. Before autoclaving, PDA was calibrated to these distinct pH levels. A 6 mm mycelial disk from the margins of 7-day-old isolates was placed individually at the center of 9 cm Petri dishes, each containing 15 mL of PDA medium. The Petri dishes, organized in triplicate for each pH condition, were then incubated in darkness at 28°C for 5 days. Post-incubation, the colony diameter was measured to ascertain growth outcomes.

STATISTICAL ANALYSIS

The present experiment was conducted utilizing a Completely Randomized Design (CRD) to ensure a balanced and unbiased assessment of the variables. The acquired data were subjected to a rigorous analysis using Analysis of Variance (ANOVA), wherein the distinction between treatment means was considered statistically significant at a probability level of $P \leq 0.05$. Further investigation involved the application of the Least Significant Difference (LSD) test, a critical tool used to discern meaningful differences among the treatment means. This approach facilitated a comprehensive understanding of the relative impact and effectiveness of the various treatments under consideration. For the generation of graphical representations, *GraphPad Prism* 8.0.1 software from Analytical Software (Tallahassee, FL, USA) was employed, alongside Microsoft Excel. These software tools ensured the creation of visual aids that effectively communicate the experimental results. Moreover, the complex task of statistical analysis was proficiently executed using Statistix Version 8.1 software. This specialized software package enabled robust statistical computations, providing a solid foundation for interpreting, and drawing insights from experimental data. Through the integration of these analytical tools and software, the research findings were effectively evaluated and elucidated, ensuring a comprehensive understanding of the experimental outcomes.

RESULTS

Identification of the pathogen causing shot-hole disease: The observed symptoms of the shot hole disease manifested as small reddish areas on both leaves and fruits, which gradually expanded to form brownish/purplish spots ranging from 3 to 10 mm in diameter. These spots exhibited a central necrotic region that eventually led to the formation of characteristic holes. On fruit surfaces, the lesions predominantly developed on the upper side and progressed to become corky and rough. The infection extended to dormant buds, resulting in their death, occasionally accompanied by the presence of a gummy exudate that conferred a varnished appearance. Twig lesions, measuring mostly 3–10 mm in diameter, were also recorded. Lesions were notably present on the fruit of cherry trees. The pathogen, identified as *W. carpophilus*, displayed consistent morphological and physiological characteristics across all isolates. Hyphae were septate, thin-walled, and branched, measuring 2.5–7.5 mm in diameter, and exhibited a sub-hyaline to light brown coloration. The conidia produced by the fungus after 7 days on PDA medium shared uniform characteristics. These conidia were spindle-shaped, with ovate apical cells and truncate basal cells. They featured 1–7 transverse septa, with a predominant occurrence of conidia possessing four septa. The color of the conidia evolved from colorless and transparent in their immature stage to sub-hyaline to golden brown, and eventually dark olivaceous to dark brown when observed collectively. Through evaluation of both morphological and physiological traits, as well as optimal growth temperatures in culture, all tested isolates were confidently identified as *W. carpophilus*. In line with proper documentation, samples of the materials have been securely stored at the Institute of Plant and Disease Management (IPDM) department in Gilgit, ensuring their availability for further study and reference.

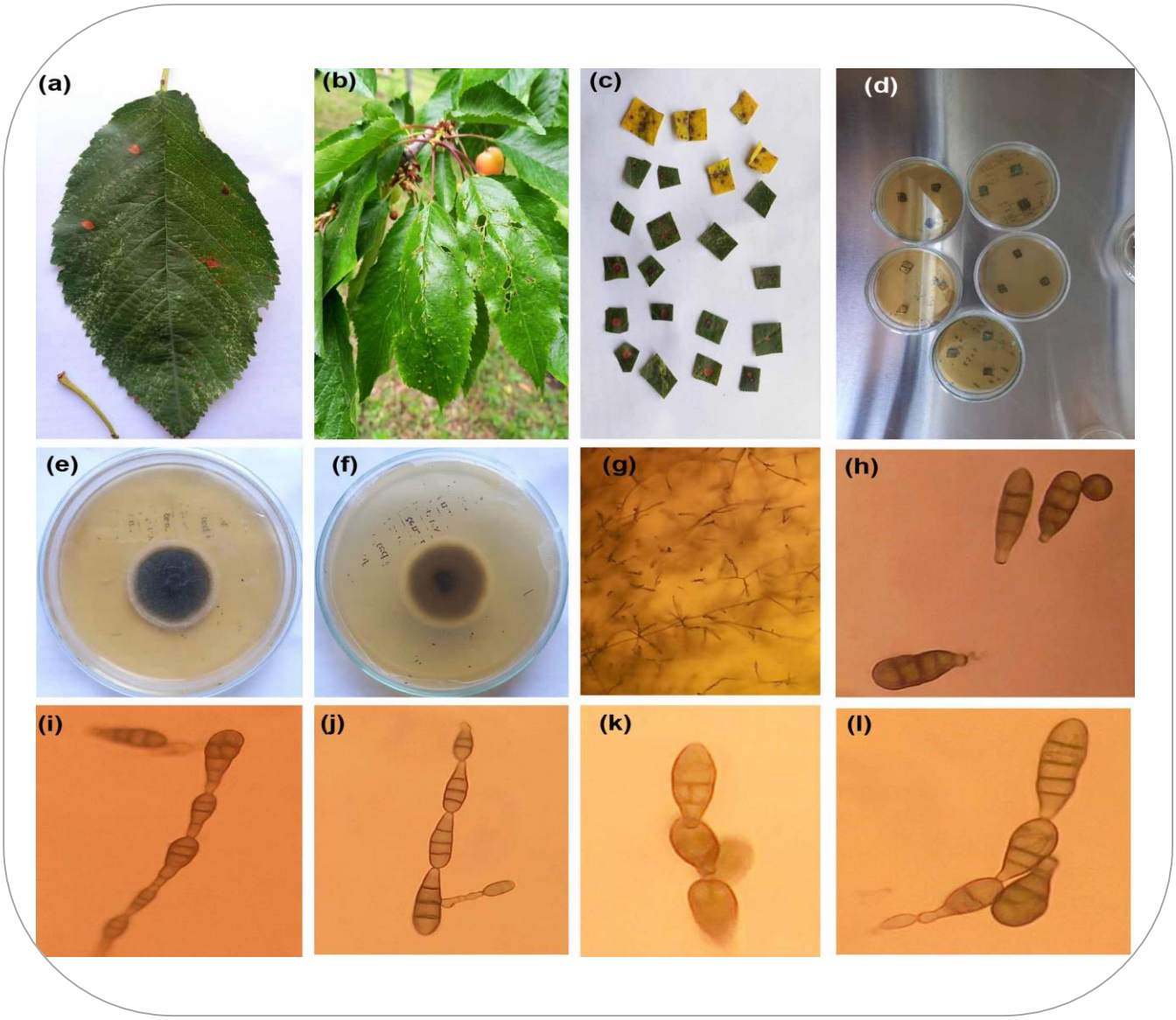

Figure 1. Shot hole fungus isolation and morphological features from affected leaves and fruits of sweet cherry. (a) Small round reddish spots/lesions observed on the surface of affected cherry leaves; (b) Gradual progression of lesions resulting in hole-like appearance; (c) Thin leaf section placed on tissue paper for drying; (d) Thin leaf section placed on PDA medium for shot hole fungal isolation; (e) Culture of shot hole fungus on PDA medium after 5 days, showing details of the front side; (f) Detailed view of the reverse side of the culture; (g) Hyaline, septate, and smooth-walled hyphae and mycelia with primordial conidia; (h-l) Brown, fusiform, smooth-walled conidia with transverse and oblique septa.

Pathogenicity test: Pathogenicity tests were performed using five isolates of *W. carpophilus*. Inoculation of sweet cherry leaves was carried out with these isolates. Following inoculation, all leaves treated with *W. carpophilus* isolates exhibited the development of circular brown lesions with pale centers within a span of 3–5 days. As the experiment progressed to 15 days post-inoculation, the entire lesion underwent collapse, resulting in the formation of a small hole. Significant variations in virulence were observed among the five *W. carpophilus* isolates when inoculated on leaves. Notably, the SRA-5 isolate displayed the lowest virulence, as indicated by its least severe impact on the plant tissue. Conversely, the isolates SRA-1, SRA-2, SRA-3, and SRA-4 exhibited the highest lesion sizes on the leaves, indicating their comparatively higher virulence levels.

Differential morphological responses to temperature variations: Morphological variations

were evident among shot hole isolates after 5 days of cultivation on Potato Dextrose Agar (PDA), incubating in incubator at different temperatures of 20°C, 24°C, and 28°C. Notable differences in growth rates were observed among isolates, with SRA-1 and SRA-4 exhibiting faster growth at all temperature regimes compared to other isolates, while SRA-5 displayed slower growth. Although colony diameters of SRA-1, SRA-2, SRA-3, and SRA-4 were comparable at 20°C and 24°C, SRA-1 exhibited the highest diameter at 24°C, while SRA-5 consistently displayed the smallest colony diameter.

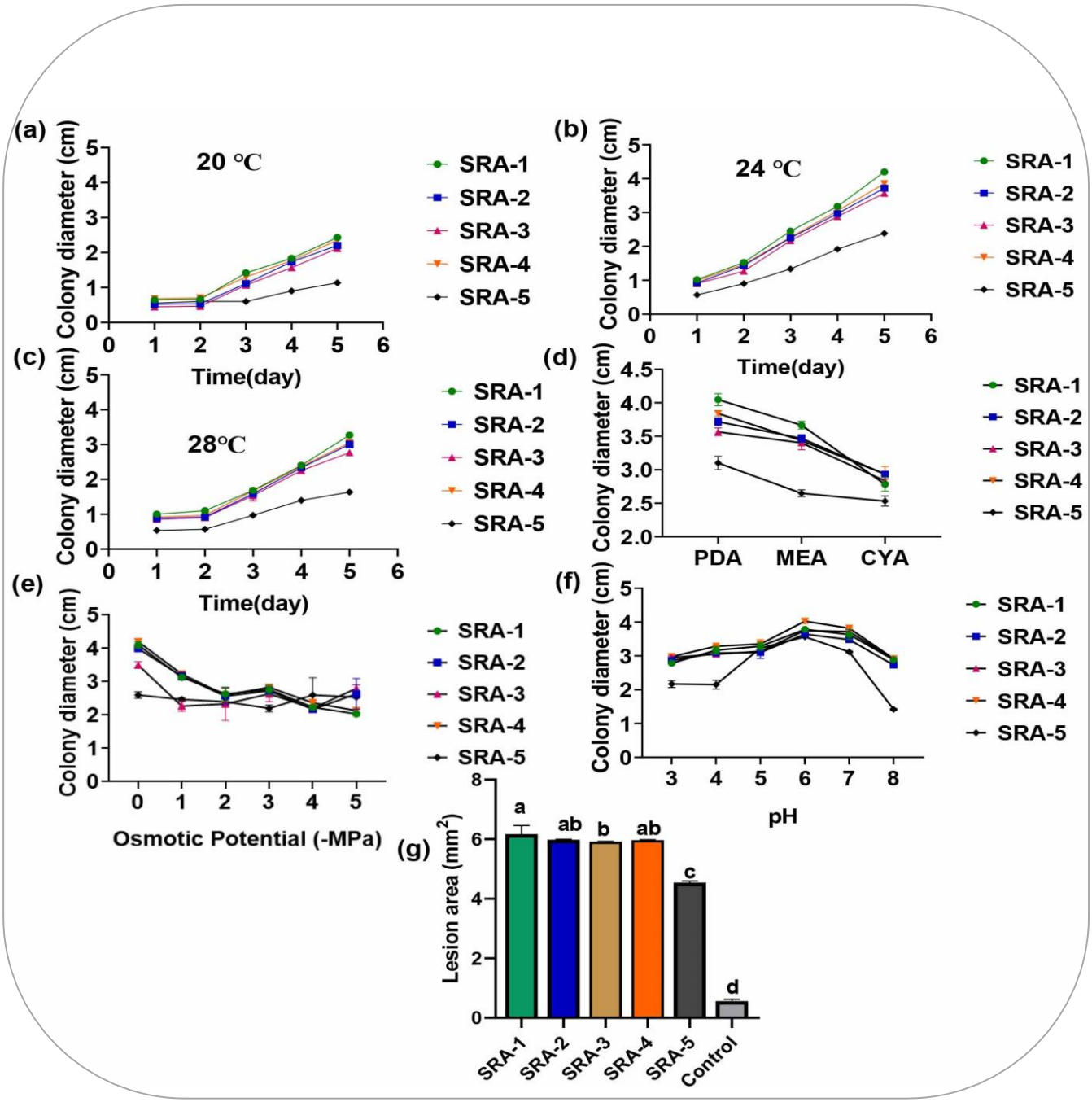

Figure 2. Growth Performance Analysis of Shot Hole Fungal Isolates under Varied Conditions. (a) Growth performance at 20°C; (b) Growth performance at 24°C; (c) Growth rate at 28°C; (d) Colony diameter variation across different culture media; (e) Growth responses under different osmotic potentials; (f) Pathogenicity test results, indicated by lesion area (mm^2)

Conidia count and biomass measurement: The response of shot hole fungal isolates to different temperatures (20°C, 24°C, and 28°C) unveiled distinct patterns in conidia count and biomass. Isolate SRA-1 consistently showed the highest conidia count and biomass across all temperatures, while SRA-5 consistently demonstrated the lowest values. Intermediate trends were observed in isolates SRA-2 to SRA-4, suggesting dynamic changes in spore count and biomass along the temperature gradient.

Growth performance and colony characteristics: Isolates SRA-1 to SRA-4 shared similar colony characteristics, displaying a velvety texture, uniform margins, and an olivaceous green color. Conversely, isolate SRA-5 exhibited distinctive attributes, including a cottony texture, uniform margins, and a light greyish color. The consistent color patterns of isolates SRA-1 to SRA-4 hint at a possible link between colony color and their superior growth performance. These contrasting characteristics in isolate SRA-5 raise the possibility of underlying physiological or genetic variations.

Table 1. Conidiation and growth of shot hole fungus isolates at different temperatures.

Isolates	Temperature (°C)							
	20		24		28			
	Conidia[1]	Biomass[2]	Conidia[1]	Biomass[2]	Conidia[1]	Biomass[2]	Texture	Color
SRA-1	20a	29a	30a	37a	36a	47a	Velvety, uniform white margins	Olivaceous green
SRA-2	9c	20c	16c	30b	20c	38b	Fluffy, uniform white margins	Olivaceous green
SRA-3	11c	24b	18c	24c	23bc	40b	Fluffy, uniform whitish margins	Olivaceous green
SRA-4	15b	19c	21b	29b	26b	39b	Fluffy, uniform margins	Olivaceous green
SRA-5	3d	8d	7d	17d	11d	24c	Cottony, uniform margins	Light greyish
LSD (P ≤ 0.05)	1.6	3.2	3.5	4.0	3.6	6.0		

[1]Conidia: × 10^5, Biomass[2]: mg, Values are means of three replicates. Different letters in the column are significantly different at (P ≤ 0.05). SRA-1-SRA-4 has almost the same colony color however SRA-5 has a different color.

Effects of pH, culture media and osmotic potential on shot hole fungus: The response of shot hole fungal isolates to varying pH, culture media, and osmotic potential was investigated. Notably, their growth exhibited distinct behavior under different osmotic potentials, indicated using varying concentrations of KCl (0-5g/L). Isolates SRA-1 to SRA-4 displayed a significant reduction in colony growth with increasing osmotic potential, reflecting their sensitivity to osmotic stress. In contrast, SRA-5's colony growth remained consistent up to 3g/L, beyond which it also experienced a decline. This divergence indicates that SRA-1 to SRA-4 is less tolerant of higher osmotic potentials compared to the relatively resilient SRA-5. Particularly, SRA-1 exhibited superior colony growth under different osmotic potentials compared to the other isolates. Similarly, the isolates' growth response was assessed across different pH levels. SRA-1 to SRA-2 displayed an increasing trend in colony growth from pH 5, peaking at pH 6 and 7, and subsequently declining. The optimal growth for these isolates was observed at pH 6 and 7, beyond which their growth began to diminish. Furthermore, the isolates' growth capabilities were examined across various culture media. Among the tested media, PDA fostered the fastest growth, followed by MEA and CYA. Notably, isolate SRA-5 exhibited a distinct preference, growing most rapidly in MEA, followed by CYA and PDA. These findings underscore the isolates' adaptability to different

environmental conditions, with some isolates demonstrating heightened sensitivity to specific factors like osmotic potential and pH, while others exhibited preferences for certain culture media. The study provides insights into the isolates' behavior and could contribute to refining cultivation practices for these shot hole fungal isolates.

DISCUSSION

The results of the study provide valuable insights into the morphological characteristics, pathogenicity, temperature responses, conidia count, biomass measurement, growth performance, and colony characteristics of the shot hole fungus, *W. carpophilus*. The consistent morphological and physiological characteristics across all isolates confirm their identification as *W. carpophilus*. Current investigation is in the line with the studies of previous researchers who also showed *W. carpophilus* as pathogen of shot hole disease (Smith *et al.*, 2009; Molnár *et al.*, 2022a; Khalilabad and Fotouhifar, 2023) who have confirmed that shot hole disease of stone fruit trees, caused by the fungus *W. carpophilus*, produces lesions on leaves and fruits. Across all the growing areas of stone fruits, this serious pathogen causes large circular purple-brown spots with chlorotic haloes on leaves. Buds and twigs are affected, too. In our study, the pathogenicity tests convincingly demonstrated that *W. carpophilus* is indeed responsible for the observed shot hole disease symptoms. The development of circular brown lesions with pale centers that progressed to hole formation on leaves post-inoculation highlights the pathogen's ability to cause significant damage to the plant tissue. The variation in virulence among different isolates underscores the genetic diversity within the pathogen population, which could be crucial in understanding disease dynamics. Previous research shows that leaf infection leads to defoliation in the most serious aspect of shot hole diseases, because severe defoliation during early fruit development can cause the young fruits to fall, and repeated defoliation weakens the trees and reduces their yield. With lesions on the petiole, the leaf is killed outright (Teviotdale *et al.*, 1999; Molnár *et al.*, 2022b). Frequently, large numbers of young leaf clusters are killed by lesions that develop on the base of the petioles. In our observations, the affected areas of the blades of mature leaves separated quickly from the non-affected tissue by abscission zones and immediately fell away. Newly formed leaves with only a few lesions dropped (Figure 1a-b), but older leaves commonly remained on the tree, despite lesions. In this study, the conidia characteristics of the isolates were similar to those of *W. carpophilus*: conidia with transversal septate (2–5), colorless and transparent when immature, turning sub-hyaline, dark olivaceous to dark brown with age reported by (Ye *et al.*, 2020). The current research endeavors into unraveling the intricate relationship between morphological variations among shot hole isolates and their responses to diverse temperature regimes, offering valuable insights into their adaptability within varying climatic conditions. Our study examines the growth rates and colony diameters at distinct temperature settings, shedding light on the unique responses exhibited by individual isolates. These distinctive behaviors imply the potential existence of isolates with inherent advantages tailored to specific temperature niches. Moreover, the assessment of differential conidia counts and biomass measurements in the context of temperature fluctuations further accentuates the malleable growth patterns of shot hole fungus. Notably, the conspicuously higher conidia count, and biomass demonstrated by isolate SRA-1 across all temperatures indicate its propensity for rapid colonization and potential dissemination. Conversely, the comparatively lower values observed for isolated SRA-5 may signify its reduced growth rate and less assertive tendencies. The observed morphological characteristics of the isolated shot hole fungus are notable, with the formation of sub-hyaline, septate, and smooth-walled mycelium.

Conclusions and future perspectives: In conclusion, shot-hole disease poses a significant and recurrent threat to cherry trees in the Gilgit-Baltistan region of Pakistan, manifesting as distinctive reddish areas on leaves and fruits, ultimately leading to characteristic holes. Despite its prevalence, critical aspects of this disease, including the identity of the causative pathogens, their pathogenicity, and their responses to various environmental conditions, have remained enigmatic. Through a comprehensive research endeavor, this study successfully identified the pathogen responsible for shot-hole disease as *W. carpophilus* and unraveled intriguing variations in pathogenicity among different isolates. Notably, SRA-5 displayed milder lesion sizes compared to SRA-1 to SRA-4, which exhibited more extensive lesions. Furthermore, the research shed light on the distinct responses of these isolates to temperature fluctuations, with SRA-1 and SRA-4 exhibiting accelerated growth

under varying temperature regimes. Conidia count and biomass measurements revealed isolate-specific growth patterns under different temperature conditions, while colony characteristics and environmental responses showcased intriguing variations among isolates. Sensitivity to osmotic potential and pH, as well as preferences for specific culture media, further elucidated the adaptability of *W. carpophilus*. Future research directions encompass genomic analysis to explore genetic composition and virulence factors, mechanistic studies for understanding molecular interactions, expanded epidemiological studies, integrated disease management, climate change impact assessment, and host-pathogen interaction studies aimed at developing disease-resistant cultivars.

Availability of data and materials: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

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