Exploration on the Mildew Conditions of *Cladosporium asperulatum* CY-H1, a Pathogenic Fungus on Tobacco Leaves

ABSTRACT

Aims: Mildew poses a significant threat to tobacco industry. This study focuses on a strain of *Cladosporium asperulatum* isolated from moldy tobacco leaves to investigate its growth under different temperature and humidity conditions, aiming to provide a theoretical basis for local prevention and control strategies against this fungus.
Methodology: Potential fungal strains were obtained from moldy tobacco leaves utilizing isolation techniques and individual colonies were cultured on PDA medium subsequently. Microscopic examination and molecular identification were employed to determine the species of the isolated strains. Isolated strain was initially inoculated with varying spore powder concentrations at water activities of 0.99, 0.95, 0.90, 0.87, 0.83, and 0.77, combined with diverse culture temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C on PDA. The growth of colony area was daily measured by ImageJ software, and spore powder yield was observed after a 7-day incubation period under these specified culture conditions.
Results:(1) colony area of isolated *C. asperulatum*CY-H1 has a rising trend with increasing culture time at consistent temperatures. Sporulation exhibited an initial increase followed by a decrease, yet overall demonstrated an upward trajectory. The maximum value was attained at a water activity of 0.95. Under identical water activity, escalating temperatures initially augmented colony area until reaching a maximum at 25°C. Similarly, spore production displayed an initial increase followed by a decrease, with the highest production occurring at 25°C. (2) Varied concentrations of initial spore suspension influenced colony growth differently. A high concentration (initially 10^6spores/mL) exhibited an earlier growth trend compared to medium (10^5spores/mL) and low concentrations (10^4spores/mL). (3) *C. asperulatum*CY-H1 did not thrive under the culture conditions of 35°C, irrespective of the initial inoculation concentration and water activity.
Conclusion:*C. asperulatum* assumes a crucial role as a predominant mold in tobacco storage environments. Effective prevention of fungal mildew in stored tobacco leaves is achieved by maintaining temperatures exceeding 35°C and sustaining water activity below 0.77. These findings highlight the significance of environmental conditions in controlling mold growth during tobacco storage.

Keywords: tobacco storage, temperature, water activity, colony area, spore powder yield

1. INTRODUCTION

Tobacco, recognized as a significant industrial and economic crop[1,2], plays a vital role as a primary raw material for cigarettes, making high-quality tobacco leaves crucial strategic resources for cigarette enterprises. The assurance of tobacco storage safety is imperative to preserve the quantity and quality of these raw materials [3]. Mildew poses a substantial threat to tobacco storage management, as molds thrive on tobacco leaves during the mildewing process, consuming sugars, proteins, starch, and other nutrients. Furthermore, these molds release green, blue, and black pigments, accompanied by a distinctive mildew

odor [4]. This not only leads to significant economic losses for tobacco farmers and cigarette manufacturers but also results in consumer dissatisfaction[5].

Temperature and relative humidity play crucial roles in mold growth [6]. Various fungi groups, including Aspergillus, Penicillium, Phoma, Alternaria, Cladosporium, and others, can contaminate tobacco leaves from stages like harvesting, processing, transportation, and storage[7]. Especially, Cladosporium species are widely distributed and are prevalent in plants, fungi, and other debris, with conidia of the Cladosporium genus being common constituents in the air [8,9]. Due to their small conidia forming branching chains, they can easily spread over long distances. Some species within this genus are phytopathogenic, causing leaf spots and lesions [10]. However, the specific Cladosporium species prevalent in tobacco storage environments remain poorly understood. Therefore, this study focuses on a strain of *C.asperulatum* isolated from moldy tobacco leaves to investigate its growth under different temperature and humidity conditions, aiming to provide a theoretical basis for local prevention and control strategies against this fungus.

2. MATERIAL AND METHODS

2.1 Tobacco Sampling and Strain Isolation

Tobacco leaf samples were gathered from a warehouse under ambient conditions at Zhejiang China Tobacco Industry Co., Ltd (Hangzhou, China), with temperatures ranging from 6°C to 35°C and humidity ranging from 49% to 94%. Collection involved the use of sterile gloves and bags to prevent contamination. Approximately 10g of tobacco leaves, sterilized with alcohol spray during weighing, were meticulously measured and placed in a sterile beaker. The leaves underwent crushing, followed by submersion in 500mL of sterile water containing 5mL of 1% Tween-80. To maintain sterility, the beaker was covered with tinfoil and securely wrapped with newspaper. An ultrasonic cleaner was employed for 2 to 3 minutes to obtain the cleaning solution. The resultant solution underwent sterile filtration using filter paper into a 250mL Erlenmeyer flask, subsequently divided into five 50mL EP tubes. Centrifugation at 8000 rpm for 10 minutes yielded a visible precipitate; the supernatant was discarded, and the precipitate collected. Dilution with 2 mL of sterile water ensued, with 1 mL of the diluted precipitate spread onto the Potato Dextrose Agar (PDA) culture medium. Incubation in a 25°C light environment (L:D = 12:12) facilitated culture growth. The emerging hyphae were then isolated and purified to obtain single colonies.

2.2Taxonomic Characterization

The isolate CY-H1 was cultured on PDA and incubated at 28°C for mycelium harvesting. Samples were processed for polymerase chain reaction (PCR) and stored at -20°C. DNA extraction was performed using the E.Z.N.A.® Soil Kit (Omega Bio-Tek).

The PCR reaction consisted of 5 μL (20 ng) of fungal DNA added to the mix with a final volume of 50 μL, comprised of 5 μL Buffer, 0.75 μL MgCl2, 0.5 μL dNTPs, 1.5 μL each primer (10 mM) ITS1 (5′ TCCGTAGGTGAACCTGCGG3′) and ITS4 (5′ TCCTCCGCTTATTGATATGC3′) [11], 0.2 μl of EX Taq DNA polymerase, and ultrapure water to complete the reaction volume. The amplification reactions were carried out in a Thermal Cycler with specific thermal conditions. The amplified fragments were visualized under UV light after separation on a 1% (w/v) agarose gel stained with gel red (Biotium company).

The DNA was quantified using a Nanodrop 3300 spectrophotometer (Thermo Scientific). The PCR amplification products were sequenced on an Illumina HiSeq 2500 platform at Shanghai Paison Biotechnology Co., Ltd. The accuracy of the nucleotide sequence was confirmed through bidirectional DNA sequencing and comparison with the NCBI databases using the Blast search algorithm. The sequences with the highest homology were selected.

2.3In-situ Mold Observation and In Vitro Culture of the Isolate CY-H1

2.3.1 Preparation of the Isolate CY-H1 Spore Solution

The mycelium of the isolate CY-H1 was inoculated onto PDA and incubated at a constant temperature of 28°C in an incubator for 7 days. Subsequently, a spore suspension of the isolate CY-H1 was prepared after the colonies had covered the plate surface. Sterilized distilled water was accurately drawn to create the spore liquid. The isolate CY-H1 spores were then harvested from the PDA using an inoculating loop. Subsequently, 40 mL of distilled water was added and the mixture was vortexed to obtain a homogeneous spore liquid. The spore liquid was diluted as necessary to achieve concentrations of 10^6spores/mL, 10^5spores/mL, and 10^4spores/mL, and stored at 4°C for future use.

2.3.2 Growth of Isolate CY-H1 under Varied Conditions

The PDA culture medium was prepared by mixing PDA powder with ultrapure water at a ratio of 43g:1000mL. Different water activities were achieved by adding varying proportions of glycerol, specifically 0%, 19.9%, 24.5%, 29.1%, 38%, and 48%, corresponding to water activities of 0.99, 0.95, 0.90, 0.87, 0.83, and 0.77 respectively. The culture medium was sterilized in an autoclave, distributed into petri dishes with 3 replicates for each condition, and stored at 4°C. Subsequently, the isolate was subjected to different temperature settings, with constant temperatures of 15°C, 20°C, 25°C, 30°C, and 35°C in an incubator.

2.3.3 Effect of Water Activity and Temperature on Growth

Six groups were created based on sixwater activity gradients (0.99, 0.95, 0.90, 0.87, 0.83, 0.77), which included five temperature gradients (15°C, 20°C, 25°C, 30°C, 35°C) per water activity in a sterile workbench. Each group replicates for three times. A filter paper (0.6cm diameter) was placed in the center of each culture medium, and 3μL of the prepared CY-H1 spore solution (10^6 spores/mL, 10^5 spores/mL, and 10^4 spores/mL) was pipetted onto the filter paper. The cultures were incubated for 7 days, recording the strains' growth daily by capturing images. ImageJ software was used to measure the colony area, facilitating comparison of strain growth at different water activities.

2.4 Sporulation Quantifica

Following 7-day incubation, 10mL centrifuge tubes corresponding to the number of petri dishes were filled with 5mL distilled water. Samples from strains cultured under various conditions were taken from the petri dishes using a hole punch. The samples were further collected from the dish center and edge, then mashed with tweezers before vortexing and sonicating. The sporulation amount was calculated using a hemocytometer under an optical microscope, with the average value determined.

2.5Statistical Analysis

Multiple groups of data from ImageJ software were analyzed using one-way analysis of variance (ANOVA) with Tukey's test. All analyses were conducted in the latest version of

Data Processing System (DPS) software [12]. All data were presented as the mean ± standard deviation. P<0.05 was considered to indicate a statistically significant difference.

3. RESULTS

3.1Taxonomic Characterization of strain

The strain CY-H1, isolated and purified from tobacco leaves, are presented in Fig. 1-A (a, b, c). The hyphae exhibit branch-like structures, are yellow-brown in color, and display dense growth. The ITS sequence amplified by the primer pair showed complete similarity (100%) to *Cladosporium asperulatum* (KF850390.1) in the GenBank database through BLAST comparison, and has been deposited at the National Collection Center for preservation (with collection numbers CCTCC M 20211690). The growth behavior of *C.asperulatum* CY-H1 on tobacco leaves under 90% relative humidity conditions was illustrated in Fig. 1-B. Initially, colonies inoculated on the leaf surface appeared as black flakes with dense hyphae. By the 4th day of cultivation, the colonies developed visible white fluffy mycelium, with the surrounding tobacco leaves darkening. By the 6th day, the white fluffy hyphae on the colony turned dark brown, the number of colonies on the leaf surface increased, the colony area expanded, and the adjacent tobacco leaves shriveled and blackened, indicating the strong pathogenicity of *C.asperulatum* on tobacco leaves.

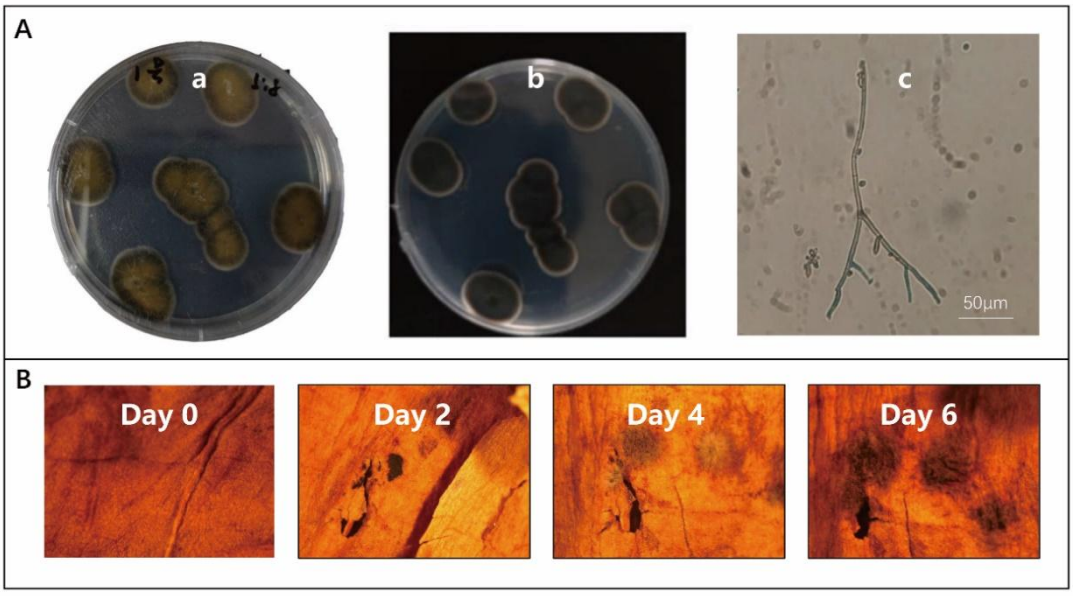

Fig. 1.Colony growth morphology of strain CY-H1 on PDA medium (A -a is a photo from the front, A-b is a photo from the back), microscopic photo (A -c) and daily growth on tobacco leaves under 90% humidity conditions (B).

3.2Effects of interaction between temperature and humidity on Cladosporium colony area under different initial inoculum concentrations of spore powder

The area of three spore powders with initial inoculation concentrations of 10^4spores/mL, 10^5spores/mLand 10^6spores/mL on the culture medium are presented in Fig. 2, 3, and 4, respectively. In Fig. 2, at a low inoculum concentration (10^4spores/mL), equivalent to 30

spores, water activity significantly influenced the colony area of isolate CY-H1. Notably, at a water activity of 0.77, no growth was observed at any culture temperature, while growth initiation at 25°C only occurred on the 7th day at a water activity of 0.83, with no growth at other temperatures. Colonies began growth after the 3rd day only at 25°C and a water activity of 0.90 or higher, showing no growth at other temperatures. Notably, no growth occurred at 35°C under various water activities, indicating optimal growth conditions at 25°C. Similar growth patterns were observed at a water activity of 0.99.

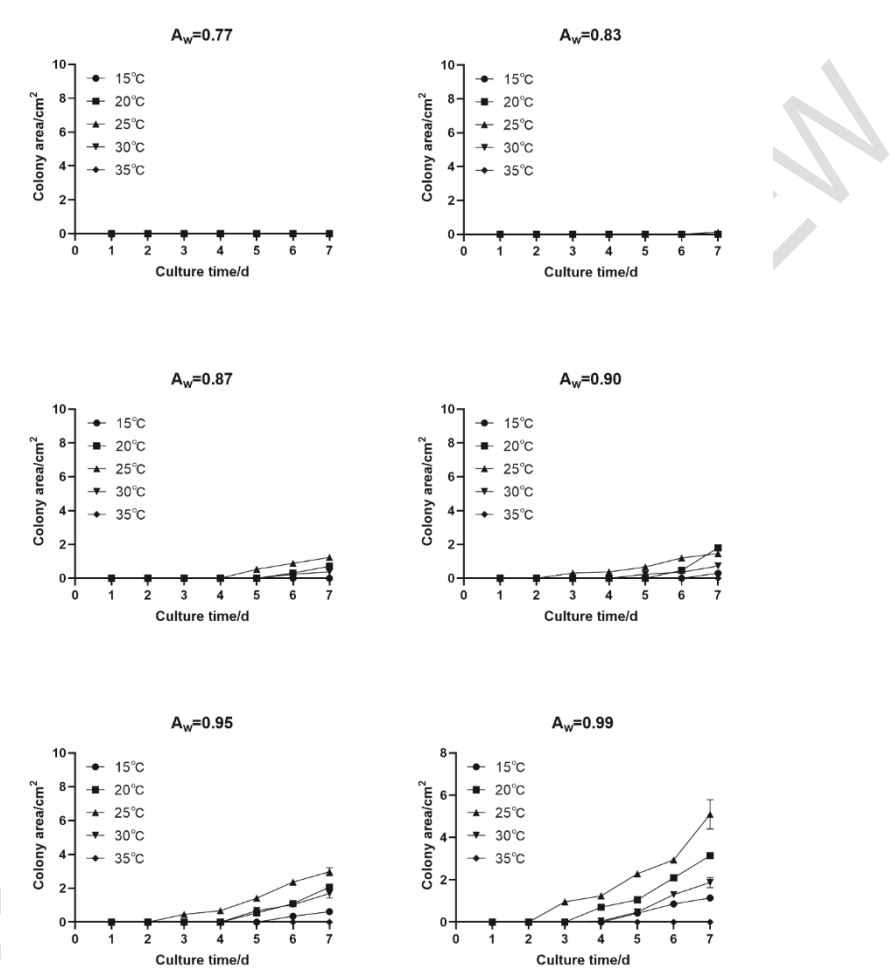

Fig. 2.Effect of the interaction between temperature and humidity on the colony area of isolate CY-H1 with an initial inoculum concentration of 10^4 spores/mL.

In Fig. 3, under a medium inoculum concentration (10^5spores/mL), equivalent to 300 spores, the isolate CY-H1 colony area was again significantly impacted by water activity. Similar to the previous scenario, growth initiation at 25°C on the 5th day at a water activity of 0.83 was observed, with no growth at other temperatures. Optimal growth was seen at 25°C and a water activity of 0.99.

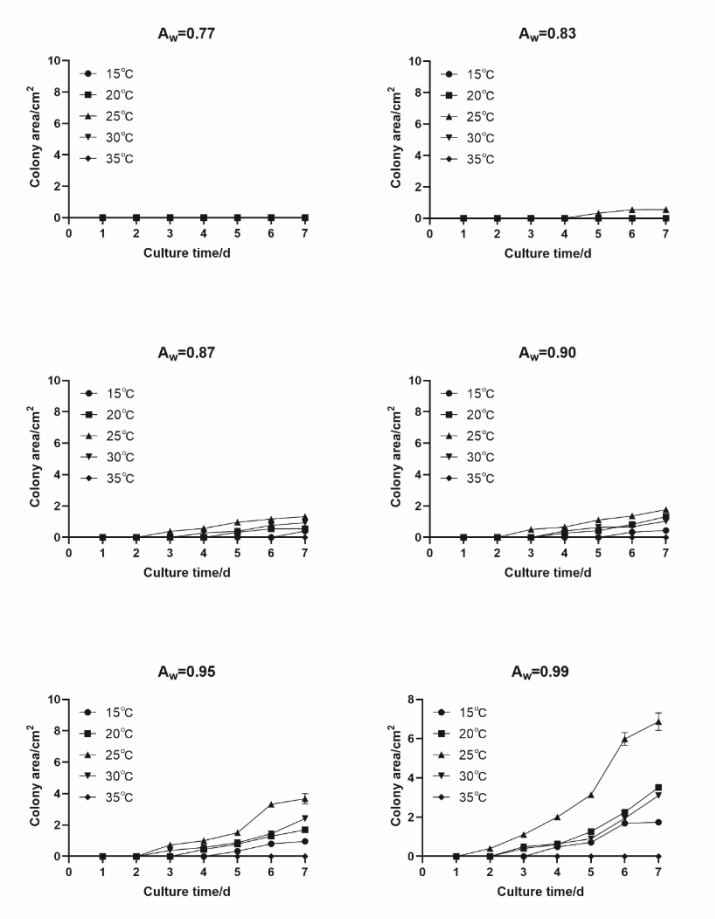

Fig. 3.Effect of the interaction between temperature and humidity on the colony area of isolate CY-H1 with an initial inoculum concentration of 10^5 spores/mL.

In Fig. 4, at a high inoculum concentration (10^6spores/mL), equivalent to 3,000 spores, water activity played a crucial role in the area of isolate CY-H1 colonies. Growth initiation occurred above 20°C on the 5th day with a water activity of 0.83, but no growth was observed at 35°C. Initial growth was only seen on the second day at temperatures exceeding 20°C and a water activity of 0.95. Optimal growth conditions were also observed at 25°C and a water activity of 0.99.

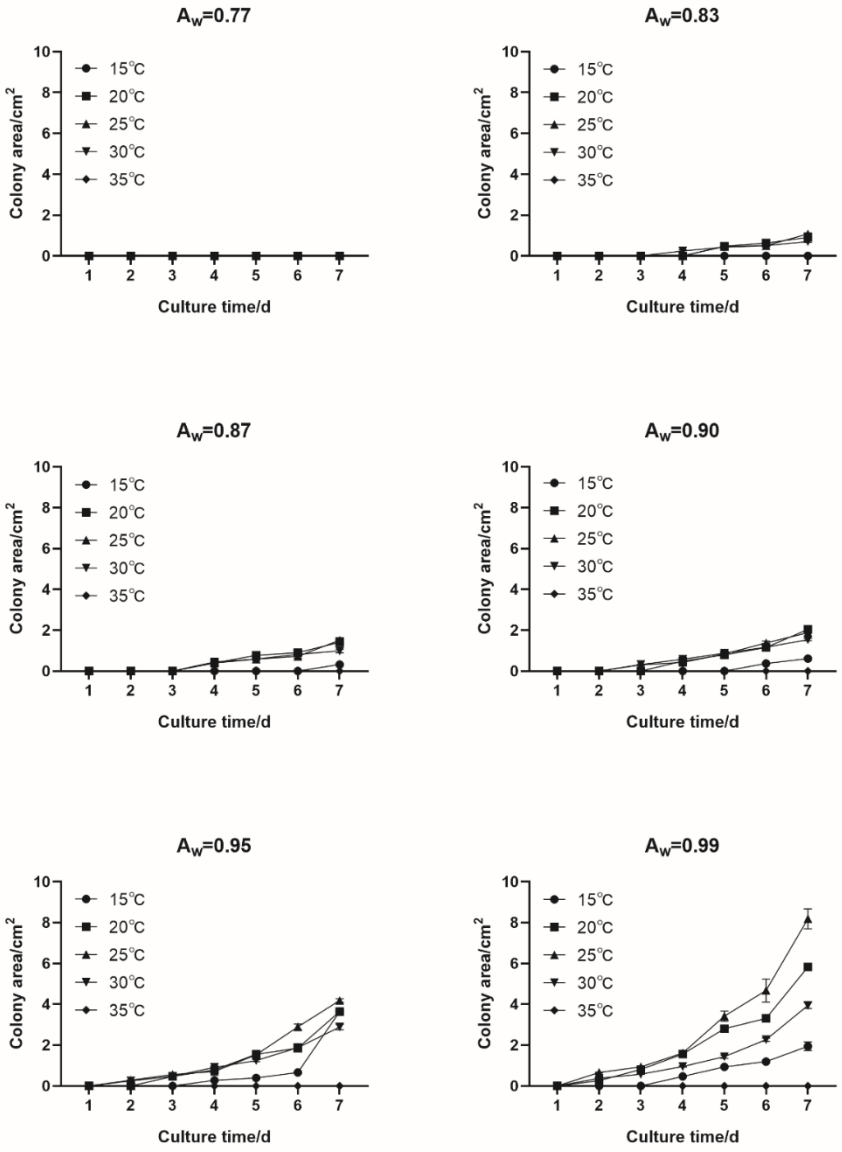

Fig. 4.Effect of the interaction between temperature and humidity on the colony area of isolate CY-H1 with an initial inoculum concentration of 10^6 spores/mL.

3.3Effects of interaction between temperature and humidity on sporulation of isolate CY-H1

Fig. 5 illustrated the relationship between spore production after seven days of cultivation, various initial inoculation concentrations of isolate CY-H1 spore powder, and different combinations of water activity and temperature. The growth of isolate CY-H1 thrives at 25°C, while growth is enhanced with a water activity exceeding 0.90. With prolonged culture time at the same temperature, the colony area exhibits a rising trend, and spore production initially increases, then decreases, but maintains an overall upward trajectory, peaking at a

water activity of 0.95. Similarly, under constant water activity, increasing temperature initially boosts colony area up to the maximum at 25°C, and spore production peaks at the same temperature before declining. Consequently, the optimal growth conditions for isolate CY-H1 are a temperature of 25°C and a water activity greater than 0.95.

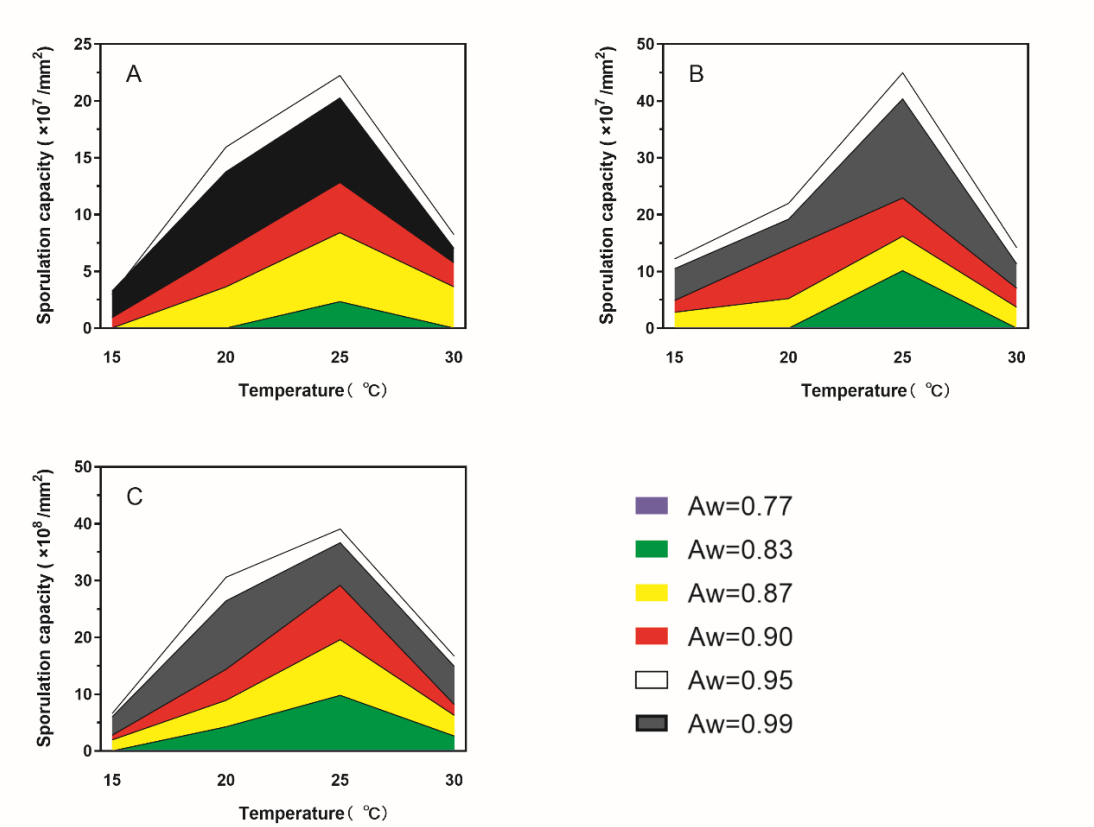

Fig. 5.Effects of different initial inoculation concentrations of isolate CY-H1 spore powder yield. A indicates the initial inoculum concentration of 10^4 spores/mL, B indicates the initial inoculum concentration of 10^5 spores/mL, C indicates the initial inoculum concentration of 10^6 spores/mL.

4. DISCUSSION

Cladosporium has been identified in aquatic environments within extreme climates, such as the Tibetan Plateau [13]. *Cladosporium*, along with *Aspergillus*, *Penicillium*, and *Talaromyces*, is considered among the most common genera indoors, with some species predominant in ambient conditions. *Cladosporium* species are among the most abundant fungi in both outdoor and indoor air[14,15], impacting agricultural yields through various fruit rots caused by species within the Cladosporiaceae genus[16,17]. *C.asperulatum* CY-H1 was isolated and verified to cause mold on tobacco leaves in the study.

Therefore, distinct growth patterns of colonies were observed with varying initial spore suspension concentrations. For instance, at a water activity of 0.99 and an initial concentration of 10^6 spores/mL, isolate CY-H1 displayed growth starting on the third day at 15°C, while growth at other temperatures commenced on the second day. With an initial

concentration of 10^5spores/mL, growth initiation varied; culture at 15°C started on the fourth day, while 20°C and 30°C cultures started on the third day, and 25°C cultures began on the second day. Furthermore, for an initial concentration of 10^4spores/mL, growth onset differed with cultures at 15℃ starting on the fifth day, those at 20℃ and 30℃ on the fifth day, and those at 25 ℃ on the third day, indicating an inverse relationship between initial concentration and growth initiation.

Notably, isolate CY-H1 did not thrive at 35°C under various concentrations and water activities, implying unsuitability for growth at this temperature. This data could be valuable for controlling tobacco leaf mildew. Additionally, fungal colonies did not develop at a water activity of 0.77, aligning with previous findings [18].

5. CONCLUSION

C.asperulatum plays a vital role as a prevalent mold in tobacco storage environments and higher initial inoculation concentrations have greater growth. Furthmore, elevated temperatures exceeding 35°C and reduced water activity less 0.77 could serve as effective physical conditions for preventing fungal mildew in stored tobacco leaves.

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