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Ghorbani Khatir, Ghasem; Aghajani, Mohammad Ali; Zolfaghari, Amir; Rabbani Nasab, Hojjatollah

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Evaluación de la agresividad y esporulación del patógeno del moho verde de los cítricos, *Penicillium digitatum*, en el norte de Irán

Ghorbani Khatir Ghasem[1*], Aghajani Mohammad Ali[2], Zolfaghari Amir[1,3], Rabbani Nasab Hojjatollah[2]

Data of the Article

[1] Department of Agriculture,
Gorgan Branch,
Islamic Azad University,
Gorgan, Iran.

[2] Agriculture and Natural Resources Research and Education.
Department of Plant Protection Research.
Center of Golestan Province.
Gorgan, Iran.

[3] Federal University of Lavras.
Department of Phytopathology.
MG, 37203-202, Brasil.

***Contact address:**

Department of Agriculture,
Gorgan Branch,
Islamic Azad University,
Gorgan, Iran.
Phone: 00989111701576
Gorgan, Iran.

Ghorbani khatir Ghasem
Aghajani Mohammad Ali

E-mail address: maaghajanina@yahoo.com

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Abstract

Green mold, caused by *Penicillium digitatum*, is the most destructive postharvest disease in citrus, leading to major economic losses globally. This study aimed to assess the epidemiological traits, pathogenicity, and aggressiveness of *P. digitatum* isolates from citrus fruits in Northern Iran. A total of 150 citrus samples were collected from orchards and warehouses across three regions. Isolates were purified using the single-spore method and examined microscopically. Pathogenicity tests involved inoculating oranges with spore suspensions, recording infection frequency (IF), lesion area (LA), sporulation capacity (SC), incubation period (IP), and latent period (LP). Data analysis included ANOVA, Pearson correlation, regression, and cluster analysis. All 88 isolates showed 100 % pathogenicity. LA ranged from 40-80 %, and SC from 7.25×10^7 to 2.37×10^8 spores. The average IP and LP were 2.3 and 5.4 days, respectively. A strong correlation (r = 0.8) was found between SC and the composite aggressiveness index (CAI), while IP and LP were inversely correlated with CAI. Cluster analysis grouped isolates into four categories based on aggressiveness, indicating genetic diversity without geographic clustering. These findings demonstrate the high aggressiveness of *P. digitatum* isolates in Northern Iran and underline the urgent need for targeted postharvest management strategies. This research contributes to understanding the pathogen's behavior and supports future development of region-specific control measures.

Resumen

El moho verde, causado por *Penicillium digitatum*, es la enfermedad poscosecha más destructiva en los cítricos, causando importantes pérdidas económicas a nivel mundial. Este estudio evaluó las características epidemiológicas, la patogenicidad y la agresividad de aislados de *P. digitatum* en frutas cítricas del norte de Irán. Se recolectaron 150 muestras de cítricos en huertos y almacenes de tres regiones. Los aislados se purificaron mediante el método de esporas únicas y se examinaron microscópicamente. Las pruebas de patogenicidad consistieron en inocular naranjas con suspensiones de esporas, registrando la frecuencia de infección (IF), el área de lesión (LA), la capacidad de esporulación (SC), el período de incubación (IP) y el período latente (LP). El análisis de datos incluyó ANOVA, correlación de Pearson, regresión y análisis de conglomerados. Los 88 aislados mostraron una patogenicidad del 100 %. LA varió entre 40-80 %, y SC entre 7.25×10^7 y 2.37×10^8 esporas. Los promedios de IP y LP fueron 2.3 y 5.4 días. Se observó una fuerte correlación (r = 0.8) entre SC y el índice compuesto de agresividad (CAI), mientras que IP y LP se correlacionaron inversamente con CAI. El análisis agrupó los aislados en cuatro categorías según su agresividad, revelando diversidad genética sin agrupamiento geográfico. Estos hallazgos destacan la alta agresividad de los aislados de *P. digitatum* en el norte de Irán y la necesidad urgente de estrategias de manejo poscosecha. Este estudio proporciona una base para futuras investigaciones epidemiológicas y medidas de control específicas.

Introduction

Iran is one of the world's top ten producers of citrus, with approximately 276,000 hectares under cultivation and an annual output of around 4 million tons of citrus fruits[1]. However, postharvest diseases, particularly green mold caused by *P. digitatum*, significantly threaten fruit quality and marketability[2]. Green mold is the most common and economically damaging postharvest citrus disease globally, with reported losses ranging from 10 to 80 % depending on region and management practices[2,3]. The fungus produces abundant airborne spores that contaminate orchards, packinghouses, storage facilities, and transportation systems[4]. Several studies have highlighted the dominant role of *P. digitatum* in postharvest fruit rot. In Spain, *Penicillium* species account for 55-80 % of decay cases, while in South Africa, green mold was responsible for 75 % of fruit rot in exported citrus[5,6]. Fruit wounds sustained during harvesting serve as entry points for the pathogen, especially under warm and humid conditions, although fungicide treatments can reduce infection rates to as low as 2-4 %, untreated fruits may suffer 15-30 % decay[7].

Despite global attention to the epidemiology and control of *P. digitatum*, there is a lack of data from Iran, especially on the aggressiveness and sporulation potential of local isolates. Previous studies in the country have largely focused on fungicide efficacy rather than the biological characteristics of the pathogen.

Therefore, this study aimed to evaluate the epidemiological traits, pathogenicity, and aggressiveness of *P. digitatum* isolates collected from citrus-producing regions in Northern Iran. Understanding local pathogen variability is essential for developing effective, region-specific disease management strategies and reducing postharvest losses. The results of this research can serve as a foundation for future epidemiological studies and the formulation of targeted control measures in Iran's citrus industry.

Materials and methods

Sample collection and fungal isolation. A total of 150 citrus fruit samples exhibiting green mold symptoms were randomly collected from orchards and storage warehouses across three citrus-producing regions of Northern Iran: Gorgan (eastern), Sari and Ghaemshahr (central), and Ramsar and Tonkabon (western). Samples were transported under cold conditions and processed within 24 h. *P. digitatum* was isolated on Potato Dextrose Agar (PDA). To ensure purity, single-spore isolation was performed as previously described by Ho & Ko[8] and Zhang et al.[9].

Purification and morphological identification. After incubation at 25° C for 7 days, colonies were examined for typical green sporulation and transferred to fresh PDA plates. Morphological identification was based on colony color, texture, and microscopic features of conidiophores and conidia, using lactophenol cotton blue staining following Pitt & Hocking[10] and methods used in recent studies Desouki et al.[11].

Pathogenicity assay. Pathogenicity tests were conducted on surface-disinfected oranges. Fruits were washed, sterilized with 95 % ethanol, and wounded (1×2 mm). A 100 µL aliquot of a 5×10^6 spores mL^{-1} suspension was inoculated into each wound. Inoculated fruits (n=10 per isolate) were incubated at 25° C in high-humidity chambers for 7 days This protocol was adapted from Wang et al.[12].

Assessment of disease indices:

Frequency infection index. The infection frequency index (IF) is assessed daily, starting from the initiation of inoculation until the completion of rot pro-

gression. This index is computed as a percentage by counting the number of oranges exhibiting visible signs or symptoms of contamination among the total number of infected oranges for each pure isolate across 10 repetitions.

The IF can be calculated according to the number of infected oranges, if the number is between 0-9, the IF will be 0-100 %, respectively as described by Gonzalez-Candelas[13].

Lesion area (necrosis, rot). The necrosis area index (LA) is determined daily, starting from the time of inoculation until the end of the lesion. This index is assessed through visual estimation, based on the percentage of infected fruit tissue area in relation to the total area of the fruit tissue. The common approach involves measuring the length and width of the damage using a ruler (mm), and visually estimating the proportion of the infected tissue area compared to the total surface area of the fruit skin. This index is recorded for each repetition[14].

Sporulation capacity (SC). Is determined by quantifying the number of spores or conidia formed within each lesion. To accomplish this, starting from the time of inoculation and continuing daily until the completion of lesion, the area of sporulation within the lesion is visually measured using a ruler (mm). When the sporulation area remains unchanged for three to five consecutive days, the colony from each orange fruit is transferred in its entirety into a beaker (50 mL) of sterile distilled water. The contents are thoroughly agitated, ensuring complete dispersion. Subsequently, a 10 µL droplet of the spore suspension is extracted using a micropipette and dispensed into the hemocytometer slide cells. The number of spores is then counted using an optical microscope. To determine the spore density per unit volume and ensure accuracy, a cell count slide and an optical microscope with 40X magnification were utilized. To enhance reliability, the spore count was repeated three times[15].

Incubation period (IP). Can be calculated by determining the minimum time interval from the initiation of inoculation to the appearance of the initial disease symptoms (rot) on each fruit. Therefore, following the initiation of inoculation, the samples were observed twice daily, in the morning and evening, using a manual loupe. Once rot symptoms were detected on samples, the number of days from the commencement of inoculation to the manifestation of the lesion was recorded[16].

Latent period (LP). Refers to the minimum time interval from the initiation of inoculation to the onset of the first sporulation on each infected fruit. Accordingly, it is necessary to observe the samples twice daily, in the morning and evening, using a manual loupe. In the case of observing colony cells in the lesion area, a closer examination should be conducted using a stereomicroscope. Once pathogenic sporulation was confirmed on each sample, the number of days from the initiation of inoculation to the appearance of sporulation is recorded[16].

Composite aggressiveness index (CAI). Represents the invasive potential of isolates under ideal conditions and is calculated using the following formula[17]: Higher CAI values represent more aggressive isolates, adapted from Flier & Turkensteen[18].

$$\mathbf{CAI} = \frac{\mathbf{IF} * \mathbf{LA} * \mathbf{SC}}{\mathbf{IP} * \mathbf{LP}}$$

CAI is the composite aggressiveness index, IF is infection frequency, LA is Necrosis Area, SC is the sporulation capacity, IP is the incubation period, and LP is the latent period. Once the CAI value was calculated, the isolates were categorized based on their level of invasion.

Statistical analysis. Descriptive statistics (mean, min, max, SD, CV) were calculated for IF, LA, IP, LP, and SC using Statgraphics Centurion v.19. One-way ANOVA was applied to assess significant differences among isolates. Pearson correlation coeffi-

cients were calculated to evaluate relationships between variables. Regression analysis was used to generate indicator models. Cluster analysis was performed using Euclidean distance and average linkage to classify isolates based on aggressiveness[19].

Results

Infection frequency (IF). A total of 88 isolates of *P. digitatum* were obtained from citrus samples collected across the studied regions. All isolates presented pathogenicity, with an IF of 100 % in inoculated oranges Figure 1 illustrates representative symptoms of green mold disease on inoculated oranges, showing extensive lesion development and dense sporulation seven days after inoculation.

Figure 1 Symptoms of green mold (Penicillium digitatum) infection on artificially inoculated oranges under controlled conditions

The figure shows widespread lesion development and sporulation on the fruit surface, the left panel seven days after inoculation, the right panel shows two weeks after inoculation. All isolates exhibited 100 % infection frequency, with varying levels of lesion expansion and sporulation density.

Table 1 Summary statistics

	LA	IP	LP	SC	CAI
Average	55.02	2.31	5.41	1.32E8	6.38E10
Coeff. of variation (%)	15.44	20.46	9.19	20.28	45.36
Standard error	.40	.02	.02	1.27E6	1.38E9
Minimum	40.0	2.0	4.0	7.25E7	1.73E10
Maximum	80.0	4.0	6.0	2.37E8	2.08E11
Range	40.0	2.0	2.0	1.65E8	1.90 E11
Standard skewness	6.17	8.21	2.54	4.57	7.63
Standard kurtosis	-.40	-3.16	-7.59	2.48	5.63

Descriptive statistics. For lesion area (LA), incubation period (IP), latent period (LP), sporulation capacity (SC), and composite aggressiveness index (CAI) are summarized in Table 1.

LA ranged from 40.0 to 80.0 %, with an average of 55.02 % (CV = 15.44 %, SE = 0.40). IP varied between 2.0 and 4.0 days, average 2.31 days (CV = 20.46 %, SE = 0.02). LP ranged from 4.0 to 6.0 days, average 5.41 days (CV = 9.19 %, SE = 0.02). SC spanned 7.25×10^7 to 2.37×10^8 spores, average 1.32×10^8 (CV = 20.28 %, SE = 1.27×10^6). CAI values extended from 1.73×10^{10} to 2.08×10^{11}, mean 6.38×10^{10} (CV = 45.36 %, SE = 1.38×10^9).

Distribution and outliers

Figures 2 and 3 display the average index values and box-and-whisker plots, respectively. Outliers were found in SC and LA but none in IP or LP, confirming data distribution integrity.

Figure 2 The average values of the CAI, SC, LP, IP, and LA indices were calculated for a total of 88 isolates of *P. digitatum*

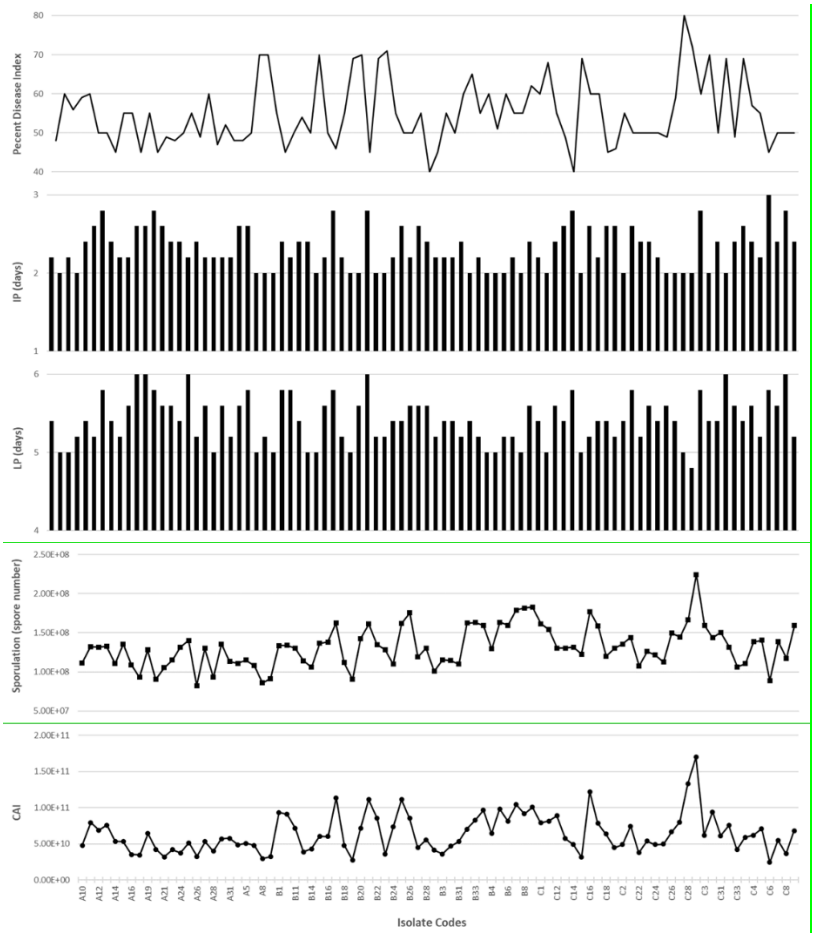

Figure 3 Box and whisker plots illustrating the studied indicators

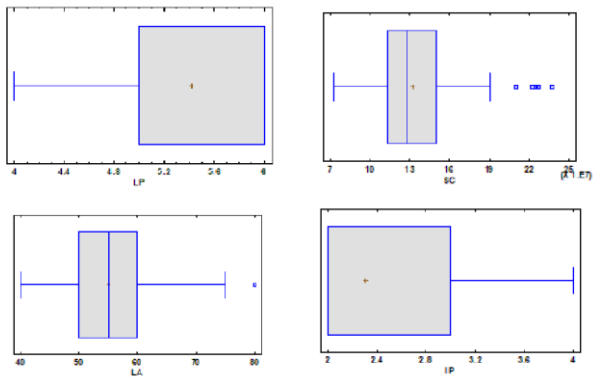

Figure 4 Correlation results

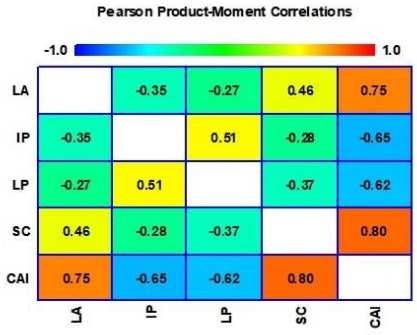

Figure **4** shows Pearson's correlation results: i) SC-CAI (r = 0.80) and LA-CAI (r = 0.75) are strong positive correlations. ii) IP-CAI and LP-CAI show strong negative correlations (r ≈ -0.65, -0.62). iii) LA correlates moderately with SC (r ≈ 0.46) and weakly negatively with LP (r ≈ -0.27).

Analysis of variance (ANOVA)

Table 2 presents ANOVA results: isolate identity (factor A) significantly affected all indices ($p \leq 0.01$), while replication (factor B) had no significant effect demonstrating real biological variability among isolates.

Grouping by index values

Mean comparison allowed grouping isolates into five categories for each index, though these groupings did not align with geographic distribution: i) LA groups ranged from 40-47 % (Group I) up to 70-80 % (Group V). ii) IP groups ranged from 2.0-2.2 days (Group I) to 2.8-3.0 days (Group V). iii) LP groups ranged from 4.8-5.0 days to 5.6-6.0 days. iv) SC groups ranged from 8.25×10^7 to 1.245×10^8 spores. v) CAI groups ranged from ~2.4×10^{10} to ~1.6×10^{11}.

Table 2 ANOVA results of LA - SC-IP-LP-CAI (type III sums of squares)

Source	Df	Mean square				
		LA	IP	LP	SC	CAI
A:NO ISO	87	356.78**	.35**	.45**	3.36E15**	3.47E21
B: Rep	4	.65ns	.35ns	.09ns	4.39E13ns	3.08E20
Residual	348	1.89	.19	.20	6.21E13	1.87E20
Total (Corrected)	439	-	-	-	-	-

The ANOVA results for the measured parameters (LA, IP, LP, SC, and CAI) indicated significant diversity among the data based on different factors. A significant difference was found among the isolates, while the repetition difference was not significant. The statistical probability level ($p \leq 0.01$ %), indicates a significant difference at the 99 % confidence level

Regression Analysis

As shown in Table 3 and Figure 5: i) Linear regressions of CAI on LA, SC, IP, and LP are all significant ($p < 0.0001$). ii) SC explains ~63.9 % of CAI variance (R^2), while LP explains ~38.3 %. iii) Residual plots (Figure 6) confirm appropriate model fit with no major violations.

Table 3 Regression results

x	y	P-value	R-squared	Standart error	Slope	Intercept
La	CAI	0.0000	55.64	1.93E10	2.54E9	-7.60E10
SC	CAI	0.0000	63.86	1.74E10	865.04	-5.02E10
IP	CAI	0.0000	41.92	2.21E10	3.97E10	1.55E11
LP	CAI	0.0000	38.25	2.28E10	3.60E10	2.59E11

Table 4 Classification of isolates based on the cluster test

Group	LA	IP	LP	SC	CAI	Aggressiveness
G1	71.8	2	5	1.76E8	1.27E11	Maximum pathogenicity
G2	63.7	2.0	5.2	1.56E8	9.53E10	Intruder
G3	58.0	2.2	5.3	1.46E8	7.60E10	Intruder
G4	50.5	2.5	5.5	1.18E8	4.68E10	Minimum pathogenicity

Figure 5 Regression plot between the CAI and LA (A), SC index (B), IP index (C) and LP (D)

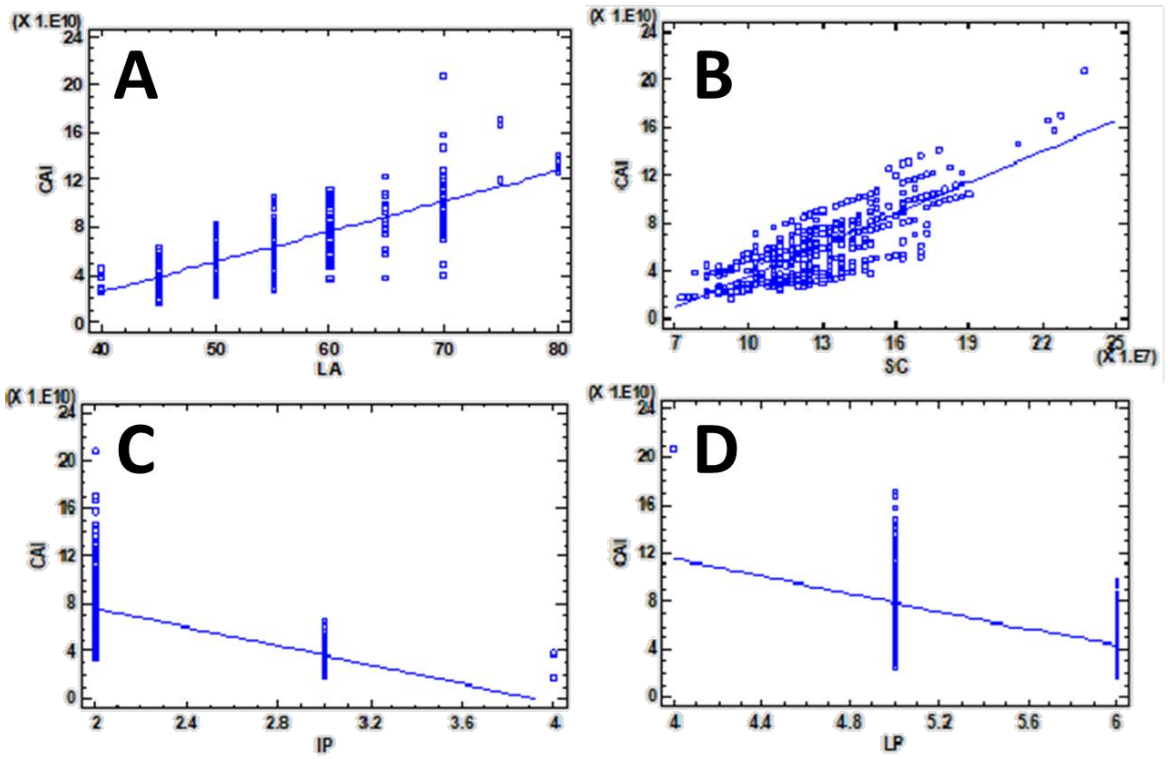

Figure 6 The residuals plot for the LA index (A), SC (B), IP (C), and LP (D)

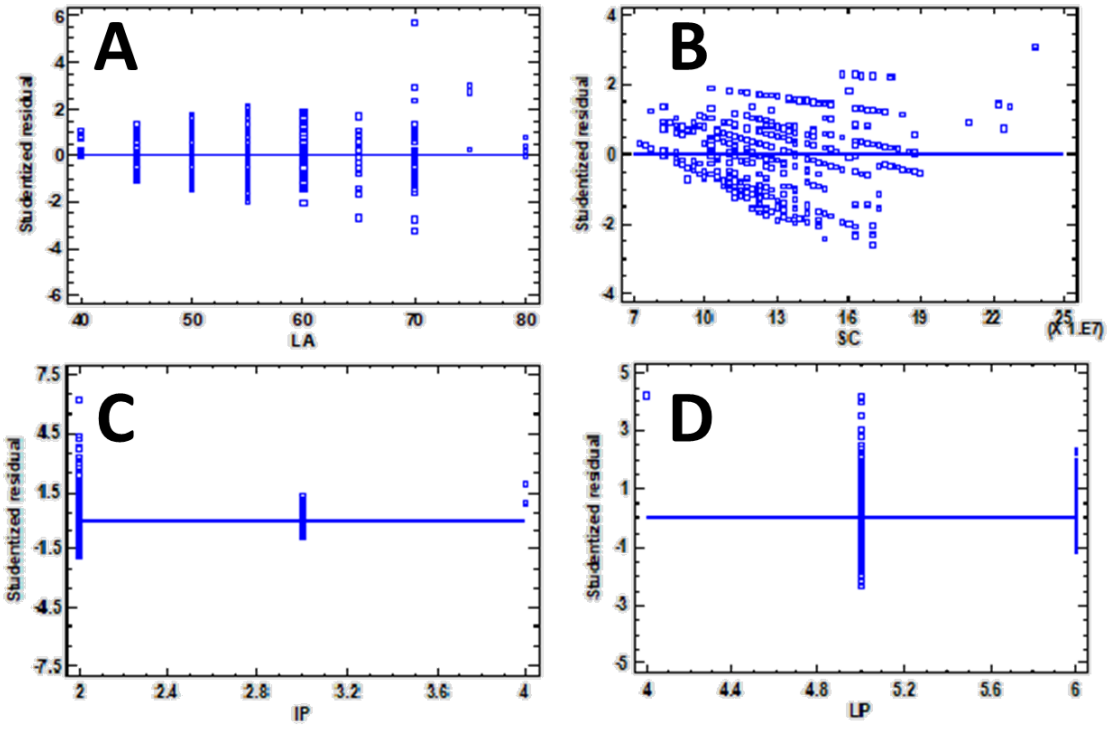

Figure 7 Classification of isolates based on the cluster test

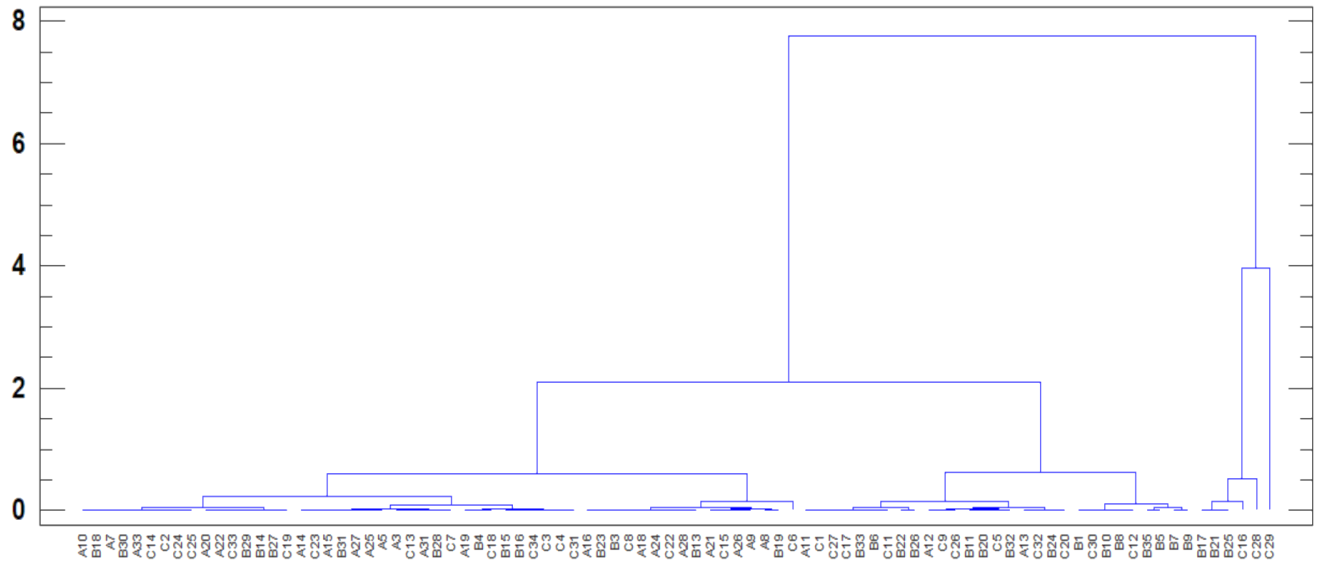

Table 5 Membership of 88 isolates of *Penicillium digitatum* under different cluster

Cluster number	Number of isolates	Percentages of isolates	Isolates
I	06	6.74	B17, B21, B25, C16, C28, C29
II	09	10.11	B9, B7, B5, B35, C12, B8, B10, C30, B1
III	20	22.47	C20, B24, C32, A13, B32, C5, B20, B11, C26, C9, A12, B26, B22, C11, B6, B33, C17, C27, C1, A11
IV	53	59.55	C9, B19, A8, A9, A26, C15, A21, B13, A28, C22, A24, A18, C8, B3, B23, A16, C31, C4, C3, C34, B16, B15, C18, B4, A19, C7, B28, A31, C13, A3, A5, A25, A27, B31, A15, C23, A14, C19, B27, B14, B29, C33, A22, A20, C25, C24, C2, C14, A33, B30, A7, B18, A10

Cluster analysis

Cluster analysis using Euclidean distance and average linkage (Statgraphics) grouped isolates into four clusters (Tables 4-5, Figure 7): i) Cluster I (6 isolates): highest aggression CAI (~1.27 × 10^{11}). ii) Cluster II (9 isolates): intermediate-high aggression CAI (~9.53 × 10^{10}). iii) Cluster III (20 isolates): intermediate aggression CAI (~7.60 × 10^{10}). iv) Cluster IV (53 isolates): lowest aggression (CAI ~4.68 × 10^{10}).

No spatial clustering by geographic region was observed.

Discussion

The main objective of this study was to examine the epidemiological aspects, pathogenicity, and aggressiveness of *P. digitatum* isolates. The findings indicate that all 88 isolates of investigated *P. digitatum* in this study were capable of causing disease and producing spores, leading to green mold on the fruit. These results highlight the pathogenic potential of the spores, particularly when they come into contact with lesions occurring by 100 % during harvesting and post-harvest stages[2,20]. Comparatively, Louw & Korsten[21], reported disease incidence rates of 100 %

in Clementine tangerine, 95 % in New Hall Novel orange, and 90 % in Eureka lemon.

Another study conducted by Neri et al.[22] evaluated the aggressiveness of four isolates of *Penicillium expansum* under laboratory conditions. Their research encompassed pear, apple, apricot, peach, strawberry, and kiwi as hosts. The results indicated that 75 % of the pathogen isolates showed the ability to cause mold in all tested hosts. Additionally, Louw & Korsten[23] and Smilanick & Sorenson[24], provided the initial report of *P. digitatum* contamination in pear fruit cultivars, which exhibited greater aggressiveness compared to *P. expansum*.

Regarding the LA index, this study revealed variations among different isolates, ranging from 40 to 80 %, with an average value of 55.02. Louw & Korsten[21], in their research comparing the pathogenicity of various *Penicillium* spp. species reported that *P. digitatum* displayed the highest growth of 80-84 mm, with an occurrence percentage of 90-100 % and disease severity ranging from 80-90 %. In contrast, for *P. italicum*, the highest growth was observed at 34-46 mm, with an occurrence percentage of 79-85 % and disease severity ranging from 20-44 %.The results of the SC index indicated that the sporulation rates varied among the isolates of *P. digitatum*. After one week, the lowest sporulation rate observed was 7.25E7 spores, while the highest was 2.37E8 spores, with an average sporulation capacity index of 1.32E8 spores. It is worth noting that the investigation of the SC index for *P. digitatum* species is limited, Holmes & Eckert[25] , and there is a lack of specific research on this aspect, Qian et al.[26] However, in a study conducted by Nava et al.[27] on the heat treatment (121° C) of *P. commune* on coffee pulp, no significant difference was found in spore production performance based on different treatment durations of 10, 20, 30, and 40 min. According to the results, the highest sporulation yield was observed at 25° C, with an average spore production of 3.7E9 spores g^{-1} for coffee pulp, Du et al.[28].

Regarding the IP index, the results showed variations among the 88 isolates of *P. digitatum*, with IP ranging from 2 to 3 days and an average value of 2.3 days. Additionally, the LP varied from 4 to 6 days among the isolates, with an average value of 5.4 days (equivalent to 135 degree- days (DD). In a comparable study, Lahlali et al.[29] reported that *P. digitatum* isolates incubated at 25° C under optimal water activity displayed a lag phase of approximately 3 days followed by rapid radial growth, with visible lesion symptoms appearing on fruits within 2 to 3 days after inoculation.

The LP is considered an important trait for invasiveness in plant pathology. Strains of pathogens with shorter LP have been found to cause more disease in previous studies[30-33]. However, the relationships between host resistance, pathogen feeding type, and LP have not been universally explored across different pathogen systems. The mentioned period typically includes an asymptomatic phase known as the latent period. Based on the findings of this research, it was observed that reducing this period and the LP index was associated with increased pathogen's aggressiveness and CAI. This finding is consistent with the research conducted by Précigout[34], who examined the variability in the LP among 53 pathogens. The study showed a wide range of this parameter, ranging from 45±7.0 DD for the necrotrophic pathogen (*Stemphylium botryosum*) to 623±65 DD for the hemibiotrophic pathogen of *Cercospora coffeicola*. Among necrotrophs, the longest LP was observed for *Ascochyta fabae*, with 134±34 DD. Furthermore, the longest incubation period among biotrophs was 322±30 DD for rusts and peanut leaf rust pathogen *Puccinia arachidis*. Hemibiotrophs generally exhibited the widest range of LP compared to biotrophs and necrotrophs, with most hemibiotrophs having an

average LP of more than 200 DD. *C. coffeicola*, in particular, exhibited an LP exceeding 600 DD. In summary, a wider range of LP was observed among hemibiotrophs compared with biotrophs and necrotrophs.

It is important to note that no prior studies have been found that specifically measure the CAI for *P. digitatum*, Lehtinen et al.[35]. The research conducted in this study represents the first evaluation of this parameter for the *P. digitatum* fungus. The lowest value observed for this index was 1.73E10, the highest value was 2.08E11, and the average composite invasion index was 6.38E10.

In a previous study by Lehtinen et al.[35], aggressiveness tests using the CAI index were conducted on *Phytophthora infestans* isolates in potato fields across Denmark, Finland, Norway, and Sweden. The study revealed differences in aggressiveness among the isolates from the different countries, although these differences were not considered significant from an epidemiological perspective. The most significant variations were observed in the LP (ranging from 89 to 185 h), SC (ranging from 100 to 1297 sporangia), and growth rate (ranging from close to zero to 6 mm day^{-1}).

Considering *P. digitatum* as a necrotrophic lesion pathogen, its high sporulation capacity is noteworthy. Research by Smilanick et al.[36] and more recently by Palou et al.[37], indicates that post-harvest rot incidence, predominantly caused by *Penicillium* spp., is more prevalent in areas with heavy rainfall. These fungi are so important as they reproduce rapidly, and their spores are ubiquitous[34]. The SC index can be considered a crucial factor in assessing the invasiveness and harmfulness of this pathogen. The CAI exhibited a high level of diversity among the tested isolates in this research, although this diversity did not show significant variation among the geographical areas studied, suggesting a genetic basis for the observed diversity[38,39].

Overall, the findings of this research demonstrate the high aggressiveness of *P. digitatum*, as all isolates were able to cause disease. The examination of various indices, including the percentage of green mold disease, fungal sporulation index, lesion percentage index, incubation period index, and latent period index, along with their direct and indirect relationships with the composite aggressiveness index, provide valuable insights into the aggressiveness and pathogenicity of *P. digitatum*. These investigations can contribute to epidemiological studies and the management of green mold disease in citrus fruits postharvest[2,40].

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Own resources were used to implement the research.

Conflicts of interest

The authors declare that this research was carried out at the Department of Agriculture, Gorgan Branch, Islamic Azad University, Gorgan, Iran and presents no conflicts of interest.

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Ethical considerations

This research is part of the Ph.D. dissertation of Ghorbanikhatir Ghasem and has been approved by

Department of Agriculture, Gorgan Branch, Islamic Azad University, Gorgan, Iran.

Research limitations

The authors note that there were no limitations in the Present research work.

Data availability

The data and materials used and analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

All authors contributed to the conception, design, and writing of the manuscript. Ghorbanikhatir Ghasem conducted the experimental work and data analysis. Aghajani Mohammad Ali contributed to the interpretation of results and manuscript editing. All authors read and approved the final version of the manuscript.

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This manuscript does not involve any studies with human participants or animals. All authors have reviewed the manuscript and consented to its submission and potential publication.

Use of artificial intelligence in writing

Artificial intelligence tools (e.g., ChatGPT by OpenAI) were used to assist in language editing and improvement of grammar and clarity. All content has been reviewed and approved by the authors to ensure accuracy and originality.

Image generation disclosure

No AI-generated images were used in this manuscript.

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