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Progress of Black Spot and Mycoparasitic Fungi in Papaya Genotypes Under Natural Inoculation Conditions

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Abstract

Papaya black spot, caused by *Asperisporium caricae*, is commonly accompanied by fungi with potential mycoparasitic activity, but their temporal occurrence across host genotypes is poorly understood. We monitored black spot and fungal colonization monthly for 12 months in 'Golden', 'Maradol', and 'Sekati' papaya plants established in a randomized complete block design with 10 replicates. Papaya black spot incidence, the incidence of leaves containing mycoparasitized lesions, total and mycoparasitism-associated disease severity, the proportion of non-colonized lesions, and the occurrence of fungal morphotypes were evaluated on intermediate and last fully expanded leaves. Repeated observations were analyzed using linear mixed models with genotype, evaluation time, and their interaction as fixed effects and block and plant as random effects. Evaluation time and the genotype × evaluation-time interaction were significant for all response variables, indicating that genotype differences varied among months. Disease severity discriminated genotypes more clearly than incidence, with 'Golden' generally showing greater severity, particularly on the last fully expanded leaf. Structures morphologically consistent with *Hansfordia pulvinata* and *Acremonium*-like fungi predominated and showed distinct temporal patterns. These results demonstrate pronounced temporal variation in disease, lesion colonization status, and fungal occurrence, but do not establish biocontrol efficacy. Culture-based and molecular identification, followed by controlled antagonism assays, is required before management recommendations can be made.

Keywords: *Asperisporium caricae*; *Carica papaya*; natural mycoparasitism; repeated measures; disease epidemiology; temporal variation

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1. Introduction

Papaya black spot, caused by *Asperisporium caricae* (Speg.) Maubl., is one of the most important foliar and fruit diseases affecting papaya [1,2]. Infection of leaves produces dark, rounded lesions that reduce the photosynthetically active area and contribute to pathogen inoculum production, whereas fruit infection causes superficial black lesions and rind hardening, reducing commercial quality and marketability [1,2]. Although papaya genotypes differ in quantitative resistance to black spot, fully resistant or immune cultivars are not currently available [3–5]. Consequently, conventional disease management relies heavily on fungicide applications [1,2,5,6], reinforcing the need for complementary strategies that can be integrated with host resistance and reduce dependence on conventional chemical control.

Biological control represents a potential component of integrated disease management when effective antagonists, appropriate application strategies, and favorable environmental conditions are identified [7–9]. Fungi associated with papaya black spot lesions include *Hansfordia pulvinata* and *Acremonium*-like fungi, which have been investigated as potential antagonists or mycoparasites of *A. caricae* [10,11]. Nevertheless, the natural occurrence of fungal structures on pathogen lesions does not, by itself, demonstrate suppression of pathogen sporulation, disease progress, or yield loss. Field observations can identify candidate organisms and characterize their ecological and temporal occurrence, whereas their antagonistic activity and biocontrol efficacy require confirmation through appropriate taxonomic characterization and controlled experimental assays.

The interaction among the host, pathogen, potential antagonist, and environment may vary markedly over time. Environmental conditions, particularly temperature, relative humidity, and rainfall, can influence the development and temporal dynamics of fungal diseases in papaya [12]. Changes in disease intensity and lesion availability throughout an epidemic may, in turn, alter the opportunities for colonization by fungi associated with *A. caricae*. Longitudinal field assessments are therefore important for characterizing the temporal dynamics of both disease and naturally occurring fungi. Moreover, because repeated observations from the same plant are not statistically independent and may exhibit temporal correlation, their analysis requires statistical approaches that account for within-plant dependence [13,14].

Previous studies have demonstrated genetic variation in papaya resistance to black spot [3–5], evaluated fungi with potential to colonize or mycoparasitize lesions caused by *A. caricae* [10,11], and demonstrated differences in the temperature requirements for the growth and sporulation of *H. pulvinata* and *Acremonium*-like fungi [15]. However, information remains limited on how black spot intensity and the natural occurrence of fungi associated with its lesions vary simultaneously over time among papaya genotypes and between leaf positions under field conditions. Longitudinal observations may help identify periods when different fungal groups are more frequently detected and determine whether their occurrence coincides with changes in disease intensity. Nevertheless, temporal coincidence among fungal occurrence, climatic conditions, and disease development should not be interpreted as evidence of causal climatic adaptation or biological control efficacy unless these relationships are tested directly.

Therefore, this study aimed to characterize the 12-month temporal dynamics of papaya black spot and the natural fungal colonization of lesions in three papaya genotypes under field conditions and to determine whether genotype differences depended on evaluation time. We also characterized the monthly occurrence of fungal morphotypes associated with mycoparasitism on two leaf positions. We hypothesized that disease intensity and fungal colonization would vary over time and that their temporal patterns would differ among papaya genotypes.

2. Material and Methods

2.1. Experimental Site and Experimental Design

The experiment was conducted in a rural area of the municipality of Mimoso do Sul, Espírito Santo State, Brazil (20°54′04.6″ S, 41°31′48.4″ W). A randomized complete block design was used, with ten blocks and three papaya genotypes differing in their level of resistance to black spot: ‘Golden’, considered more susceptible, and ‘Maradol’ and ‘Sekati’, which exhibit intermediate levels of partial resistance [3].

Each experimental unit consisted of one plant, resulting in 30 experimental plants. Plants were arranged in single rows at a spacing of 2.0 × 1.5 m. Crop-management practices were performed according to the recommendations of Marin et al. [16], except

that no fungicides were applied during the experiment. Transplanting was performed in February 2016. Disease and mycoparasitism assessments were conducted monthly for 12 consecutive months, from August 2016 to July 2017, after the appearance of black spot symptoms.

2.2. Disease and Mycoparasitism Assessments

The same experimental plants were evaluated monthly throughout the study period. Thus, evaluation time was considered a repeated factor within each experimental plant.

Papaya black spot incidence (BSI) and the incidence of leaves containing mycoparasitized lesions (MLI) were assessed at each monthly evaluation. BSI was determined as $\text{BSI}\ (\%) = \frac{\text{number of symptomatic leaves}}{\text{total evaluated leaves}} \times 100$, whereas MLI was determined as $\text{MLI}\ (\%) = \frac{\text{number of mycoparasitized leaves}}{\text{number of symptomatic leaves}} \times 100$.

Black spot severity was visually estimated at two leaf positions: (i) the last fully expanded leaf and (ii) an intermediate leaf corresponding to the leaf attached to the first newly opened flower. Severity was estimated using the diagrammatic scale proposed by Francelino et al. [17]. For each leaf position, two severity measurements were obtained: total black spot severity and the severity corresponding to lesions showing signs of mycoparasitism. These variables were designated as total black spot severity on the intermediate leaf (BSS-IL), mycoparasitism-associated black spot severity on the intermediate leaf (BSSm-IL), total black spot severity on the last fully expanded leaf (BSS-LFE), and mycoparasitism-associated black spot severity on the last fully expanded leaf (BSSm-LFE).

2.3. Assessment and Morphological Characterization of Fungi Associated with Black Spot Lesions

The proportion of non-colonized lesions (PNCL) was derived from total black spot severity (BSS) and mycoparasitism-associated black spot severity (BSSm) according to the following equation:

$$PNLC(\%) = \frac{BSS - BSSm}{BSS} \times 100$$

PNCL was calculated separately for the two evaluated leaf positions. Accordingly, PNCL-IL represented the proportion of non-colonized lesions on the intermediate leaf, calculated from BSS-IL and BSSm-IL, whereas PNCL-LFE represented the proportion of non-colonized lesions on the last fully expanded leaf, calculated from BSS-LFE and BSSm-LFE.

Fungi associated with black spot lesions were assessed monthly, simultaneously with disease assessments. At each evaluation time, 20 black spot lesions showing signs of fungal colonization were sampled from each experimental plant: ten lesions from the intermediate leaf and ten lesions from the last fully expanded leaf. Sampling was performed using a non-destructive adhesive-tape method. A transparent adhesive-tape strip was gently pressed onto the lesion surface to remove fungal reproductive structures. Tape sections containing adhered structures were mounted in lactic acid on microscope slides and examined under a light microscope for the presence of reproductive structures, particularly conidiophores and conidia.

Morphological characterization was based on reproductive structures and diagnostic traits described in the taxonomic literature. Structures morphologically consistent with *Hansfordia* were evaluated according to Seifert et al. [18], considering the presence of branched, trichodermoid or verticillate, brown conidiophores; sympodial, denticulate, and hyaline conidiogenous cells; and dry, solitary, hyaline, aseptate conidia.

Structures morphologically consistent with *Acremonium*-like fungi were evaluated according to the morphological concepts described by Gams [19,20], Domsch et al. [21], and Perdomo et al. [22]. Diagnostic characteristics considered included hyaline, septate, and branched hyphae; simple, erect, lateral or terminal monophialidic conidiogenous cells; and

small, hyaline, unicellular conidia produced at the apex of the phialides, either in chains or mucilaginous heads.

Based on these morphological criteria, fungal structures were classified as morphologically consistent with *Hansfordia pulvinata*, *Acremonium*-like fungi, or other fungi when the observed structures did not allow reliable assignment to either of these morphological groups.

Because fungal material was obtained using a non-destructive adhesive-tape method, no fungal cultures, preserved fungal biomass, or DNA suitable for retrospective molecular analysis were available. Consequently, molecular identification could not be performed. Taxonomic assignments were therefore based exclusively on morphological evidence and should be interpreted as morphology-based identifications rather than molecularly confirmed species determinations.

The occurrence frequency of each morphological fungal group was calculated for each evaluation time based on the fungal structures detected in the sampled black spot lesions and was subsequently used to characterize temporal patterns.

2.4. Climatic Data

Relative humidity and maximum and minimum air temperatures were recorded hourly throughout the experimental period using automated data loggers (WatchDog Model 450, Spectrum Technologies, Inc., Aurora, IL, USA). Rainfall data were obtained from the CEMADEN "Café" monitoring station (station code 320020107A), located in Alegre, Espírito Santo State, Brazil, approximately 5.6 km from the experimental site. Hourly temperature and relative-humidity records and rainfall data were summarized on a monthly basis to characterize climatic conditions during the 12-month evaluation period.

2.5. Statistical Analysis

Each disease, mycoparasitism, and fungal-occurrence variable was analyzed separately using a repeated-measures linear mixed model to account for the correlation among successive observations obtained from the same experimental plant [13,14]. Genotype, evaluation time, and the genotype × evaluation-time interaction were included as fixed effects. Evaluation time was treated as a categorical factor because the objective was to characterize temporal fluctuations in black spot and mycoparasitism rather than to estimate a linear temporal trend or determine an optimum evaluation date.

Block and the experimental unit, represented by the individual plant within block, were included as random effects. Because each block contained one plant of each genotype, the experimental unit corresponded to the block × genotype combination. Plant identity (Plant_ID) was used as the repeated-measures subject, uniquely identifying each genotype × block experimental unit. The 12 monthly observations obtained from the same plant were therefore treated as repeated measurements within the experimental unit. The model was expressed as:

$$Y_{ijt} = \mu + B_i + G_j + P_{ij} + E_t + (G \times E)_{jt} + \varepsilon_{ijt}$$

where Y_{ijt} is the response observed in block i, genotype j, and evaluation time t; μ is the overall mean; B_i is the random block effect; G_j is the fixed genotype effect; P_{ij} is the random experimental-unit effect corresponding to the plant of genotype j in block i; E_t is the fixed evaluation-time effect; $(G \times E)_{jt}$ is the fixed genotype × evaluation-time interaction; and ε_{ijt} is the residual error.

Alternative residual covariance structures were evaluated to account for the dependence among repeated observations from the same plant. The structures considered were an independent residual structure (IND), a first-order autoregressive structure [AR(1)], and a heterogeneous first-order autoregressive structure [ARH(1)]. Candidate models

were fitted using maximum likelihood (ML) and compared using the Akaike information criterion (AIC). Only successfully fitted models with finite AIC values were considered for selection. The covariance structure yielding the lowest AIC was selected, and the corresponding final model was refitted using restricted maximum likelihood (REML).

Under AR(1), the residual correlation between two observations from the same plant decreased exponentially as the temporal lag between assessments increased, assuming homogeneous residual variance across evaluation times. Under ARH(1), the same autoregressive correlation pattern was retained while allowing a distinct residual variance for each evaluation time.

Percentage variables (BSI, MLI, PNCL-IL, PNCL-LFE, and fungal-occurrence frequencies) were converted to proportions and subjected to arcsine square-root transformation $\left[y' = arcsin\sqrt{y/100}\right]$ before analysis. Model-based estimated marginal means and their 95% confidence intervals were subsequently back-transformed to the percentage scale $\left[y = 100sin^2(y')\right]$ for presentation. Disease-severity variables (BSS-IL, BSSm-IL, BSS-LFE, and BSSm-LFE) were analyzed on their original scale. Missing observations were treated as missing values and were not replaced by zeros; available observations were used for model fitting.

Fixed effects were evaluated using marginal F-tests from the fitted mixed model. When the genotype × evaluation-time interaction was significant at $p \leq 0.05$, model-based pairwise contrasts among genotypes were performed separately within each evaluation time using estimated marginal means. The Holm procedure was applied to adjust for multiple comparisons. Model assumptions were evaluated using normal Q–Q plots of normalized residuals and plots of normalized residuals versus fitted values. Statistical significance was declared at $p \leq 0.05$.

All statistical analyses were performed in the R statistical environment (R Core Team, Vienna, Austria). Repeated-measures linear mixed models and residual covariance structures were fitted using the nlme package. Estimated marginal means, 95% confidence intervals, and model-based pairwise contrasts were obtained using the emmeans package. Data manipulation and organization were performed using dplyr and tibble, and diagnostic and estimated-mean plots were produced using ggplot2.

3. Results and Discussion

3.1. Climatic Conditions During the Experimental Period

The experimental period was characterized by marked seasonal variation in rainfall and temperature. Maximum relative humidity approached 100% in all months, whereas the lowest median relative humidity values occurred in August 2016 and January and February 2017. Minimum relative humidity in August was close to 22%, indicating particularly dry conditions during that month. August was also the driest month in terms of rainfall (Figure 1). Precipitation subsequently increased and reached approximately 270 mm in December 2016, followed by a decline and a second peak of approximately 110 mm in May 2017. Air temperatures ranged from approximately 8 to 33 °C, with warmer conditions from November to March and cooler conditions from May to August.

These seasonal changes are relevant to the interpretation of black spot dynamics, as temperature, relative humidity, and rainfall can influence the development, sporulation, and dispersal of pathogens responsible for papaya foliar diseases [12]. Similar associations between wetter periods and increased black spot severity have been reported under other environmental conditions [23]. Recent reviews addressing tropical fruit crops and plant diseases in Brazil have also identified temperature and moisture as important drivers of disease development and specifically recognized papaya black spot as a climate-sensitive pathosystem [24,25]. Climatic conditions may likewise influence the occurrence of fungi

associated with *A. caricae* lesions; under controlled conditions, *H. pulvinata* and *Acremonium*-like fungi showed distinct temperature responses for growth and sporulation [15]. Nevertheless, the climatic and biological variables were not modeled jointly in the present study, and these observations should therefore be interpreted as temporal associations rather than causal climatic effects.

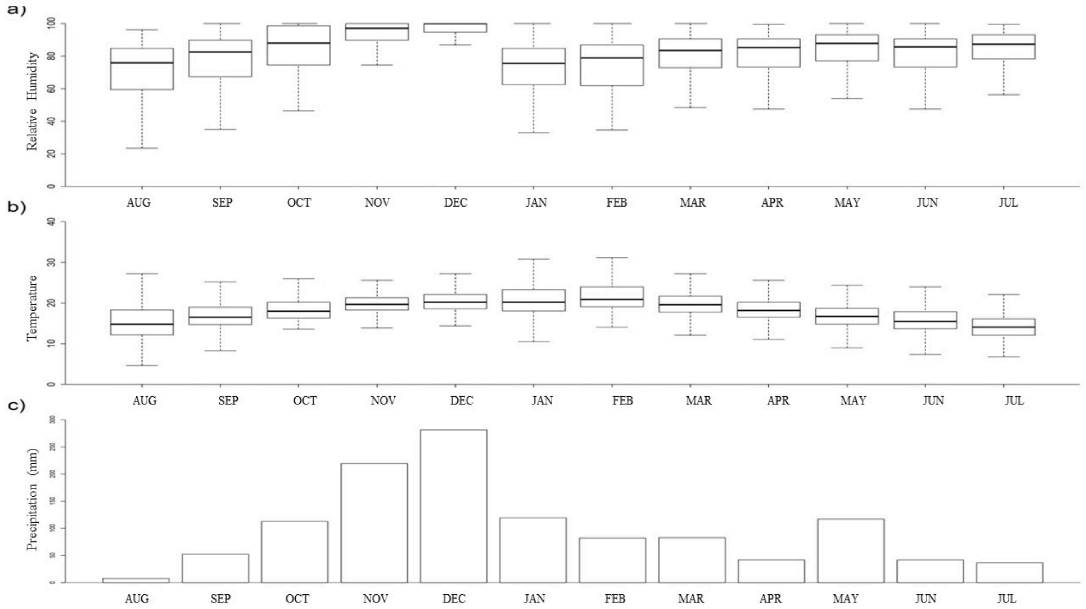

Figure 1. Meteorological conditions recorded in the experimental area in Mimoso do Sul, Espírito Santo State, Brazil, from August 2016 to July 2017: (**a**) relative humidity; (**b**) air temperature; and (**c**) precipitation.

3.2. Repeated-Measures Mixed-Model Analysis and Temporal Dependence

Repeated observations from the same plants were analyzed using repeated-measures linear mixed models. The independent residual structure (IND), the homogeneous first-order autoregressive structure [AR(1)], and the heterogeneous first-order autoregressive structure [ARH(1)] were compared using maximum likelihood and AIC, after which the selected model was refitted using REML. ARH(1) produced the lowest AIC for 10 of the 11 response variables, indicating that temporal dependence together with heterogeneous residual variances among evaluation times provided a better representation of most responses. This result reinforces the importance of explicitly accounting for within-plant dependence in longitudinal field experiments [13,14]. For the occurrence of *Acremonium*-like fungi, AR(1) and ARH(1) did not yield valid fits because the coefficient matrix was not invertible; therefore, IND was retained as the valid residual structure for this response (Table 1).

Table 1. Comparison of residual covariance structures and marginal F-test probabilities for genotype, evaluation time, and the genotype × evaluation-time interaction.

Variable	AIC IND	AIC AR(1)	AIC ARH(1)	Selected	Genotype *p*	Time *p*	G × E *p*
BSI	−467.84	−467.39	−531.82	ARH(1)	0.1622	<0.0001	<0.0001
MLI	−29.35	−27.42	−96.59	ARH(1)	0.0122	<0.0001	0.0174
BSS-IL	722.75	724.53	599.68	ARH(1)	<0.0001	<0.0001	<0.0001
BSSm-IL	693.69	695.38	573.24	ARH(1)	<0.0001	<0.0001	<0.0001
PNCL-IL	−617.45	−617.45	−717.51	ARH(1)	0.0032	<0.0001	0.0020
BSS-LFE	1106.96	1052.50	896.29	ARH(1)	<0.0001	<0.0001	<0.0001

Table 1. *Cont.*

Variable	AIC IND	AIC AR(1)	AIC ARH(1)	Selected	Genotype *p*	Time *p*	G × E *p*
BSSm-LFE	1089.62	1037.71	875.13	ARH(1)	<0.0001	<0.0001	<0.0001
PNCL-LFE	−513.87	−511.88	−579.45	ARH(1)	0.0003	<0.0001	<0.0001
H. pulvinata-like	509.98	501.38	454.18	ARH(1)	0.2938	<0.0001	0.0002
Acremonium-like	330.67	—	—	IND	0.0204	<0.0001	0.0050
Other fungi	−208.15	−207.48	−226.62	ARH(1)	0.5097	0.0010	0.0004

Note. BSI: papaya black spot incidence; MLI: incidence of leaves containing mycoparasitized lesions; BSS-IL: total black spot severity on the intermediate leaf; BSSm-IL: mycoparasitism-associated black spot severity on the intermediate leaf; PNCL-IL: proportion of non-colonized lesions on the intermediate leaf; BSS-LFE: total black spot severity on the last fully expanded leaf; BSSm-LFE: mycoparasitism-associated black spot severity on the last fully expanded leaf; PNCL-LFE: proportion of non-colonized lesions on the last fully expanded leaf. IND: independent residual structure; AR(1): homogeneous first-order autoregressive structure; ARH(1): heterogeneous first-order autoregressive structure. Percentage variables were analyzed after arcsine square-root transformation. Marginal F-test probabilities were obtained from the selected model refitted using REML. For the occurrence of *Acremonium*-like fungi, AR(1) and ARH(1) did not yield valid fits because the coefficient matrix was not invertible. AIC values should be compared only among covariance structures fitted to the same response variable.

3.3. Black Spot Incidence and Incidence of Leaves Containing Mycoparasitized Lesions

Black spot was observed in all three genotypes throughout the experimental period. For papaya black spot incidence (BSI), the genotype main effect was not significant ($p = 0.1622$), whereas evaluation time ($p < 0.0001$) and the genotype × evaluation-time interaction ($p < 0.0001$) were significant. Significant monthly contrasts occurred in December 2016 and from January to April 2017. The specific pattern varied among months: Maradol differed from Sekati in December; Maradol differed from both Golden and Sekati in January; Sekati differed from both Golden and Maradol in February; all three genotypes differed in March; and Maradol differed from Golden and Sekati in April (Figure 2A). Thus, the absence of a significant overall genotype effect should not be interpreted as uniform disease response, because genotype differences were transient and depended on evaluation time.

This temporal variation is consistent with the quantitative nature of resistance to papaya black spot. Previous field studies have identified Maradol and Sekati as useful sources of partial resistance, while Golden is generally considered more susceptible [3]. However, resistance can be expressed differently according to the disease variable and evaluation period, and reduced severity does not necessarily imply consistently lower incidence. The present results therefore indicate that BSI alone may provide an incomplete representation of genotype response when disease pressure varies substantially over time.

The incidence of leaves containing mycoparasitized lesions (MLI) was affected by genotype ($p = 0.0122$), evaluation time ($p < 0.0001$), and their interaction ($p = 0.0174$). Golden and Sekati differed in August 2016 and May 2017, whereas Sekati differed from Golden and Maradol in April 2017 (Figure 2B). MLI values were generally lower during the initial assessments and increased during later stages of the epidemic. This pattern may reflect, at least in part, greater availability of *A. caricae* lesions as substrates for fungi associated with mycoparasitism. Previous studies have demonstrated that *H. pulvinata* and *Acremonium*-like fungi can colonize black spot lesions and have potential antagonistic activity against *A. caricae* [10,11].

The increase in MLI around December coincided with increased rainfall and high relative humidity, conditions that may favor both disease development and fungal activity [12,23]. Nevertheless, because climatic variables were not included as predictors in the mixed models, this correspondence should be interpreted as temporal coincidence rather than evidence of a direct climatic effect.

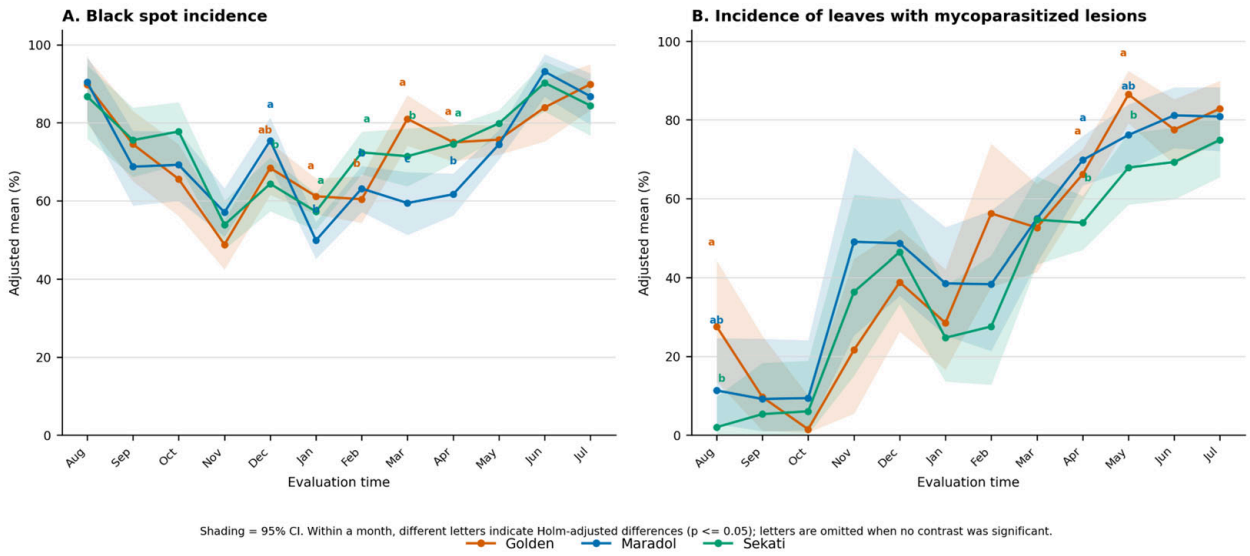

Figure 2. Model-adjusted monthly means of (**A**) papaya black spot incidence (BSI) and (**B**) incidence of leaves containing mycoparasitized lesions (MLI) for 'Golden', 'Maradol', and 'Sekati' from August 2016 to July 2017. Shaded areas represent 95% confidence intervals. Within each month, different letters indicate significant differences among genotypes based on Holm-adjusted pairwise contrasts ($p \leq 0.05$); letters are omitted when no significant genotype contrast was detected. Means and confidence intervals are shown on the back-transformed percentage scale.

3.4. Morphological Evidence for the Fungal Groups Associated with Black Spot Lesions

Microscopic examination of adhesive-tape preparations revealed reproductive structures morphologically consistent with *Hansfordia pulvinata* and *Acremonium*-like fungi. Structures assigned to the *Hansfordia* morphotype were characterized by branched, trichodermoid or verticillate brown conidiophores, sympodial denticulate conidiogenous cells, and dry, solitary, hyaline, aseptate conidia. These features agree with the diagnostic characters described for *Hansfordia* by Seifert et al. [18].

Structures assigned to the *Acremonium*-like morphotype showed septate and branched hyphae, simple erect lateral or terminal monophialidic conidiogenous cells, and small hyaline unicellular conidia produced at the apex of the phialides, either in chains or grouped at the phialide tip. These characters are consistent with the morphological concepts described by Gams [19,20], Domsch et al. [21], and Perdomo et al. [22].

The occurrence of these morphotypes on papaya black spot lesions is consistent with previous studies in which *H. pulvinata* and *Acremonium*-like fungi were recovered from lesions caused by *A. caricae* and investigated as potential mycoparasites or antagonists [10,11]. In a previous study combining morphological characterization with ITS sequencing, several isolates obtained from black spot lesions were identified as *H. pulvinata* or *Acremonium*-like fungi, supporting the biological plausibility of the morphotypes observed here [10].

Nevertheless, the taxonomic evidence obtained in the present study was exclusively morphological. The non-destructive adhesive-tape sampling method did not yield fungal cultures, preserved biomass, or DNA suitable for retrospective molecular analysis. Consequently, the assignments cannot be regarded as molecularly confirmed identifications. The fungi are therefore described as structures morphologically consistent with *H. pulvinata* and *Acremonium*-like fungi, reflecting both the diagnostic value and the limitations of the available material.

3.5. Black Spot Severity and Severity Associated with Mycoparasitized Lesions

Total black spot severity and mycoparasitism-associated black spot severity were affected by genotype, evaluation time, and their interaction on both leaf positions ($p < 0.0001$ for all effects; Table 1). On the intermediate leaf (BSS-IL and BSSm-IL), significant genotype contrasts occurred in August 2016 and from December 2016 through July 2017. On the last fully expanded leaf (BSS-LFE and BSSm-LFE), significant differences occurred from August to October 2016 and from December 2016 through July 2017; no genotype contrast was significant in November. Golden generally showed the highest adjusted severity, whereas Maradol and Sekati tended to exhibit lower values, particularly on the last fully expanded leaf (Figure 3).

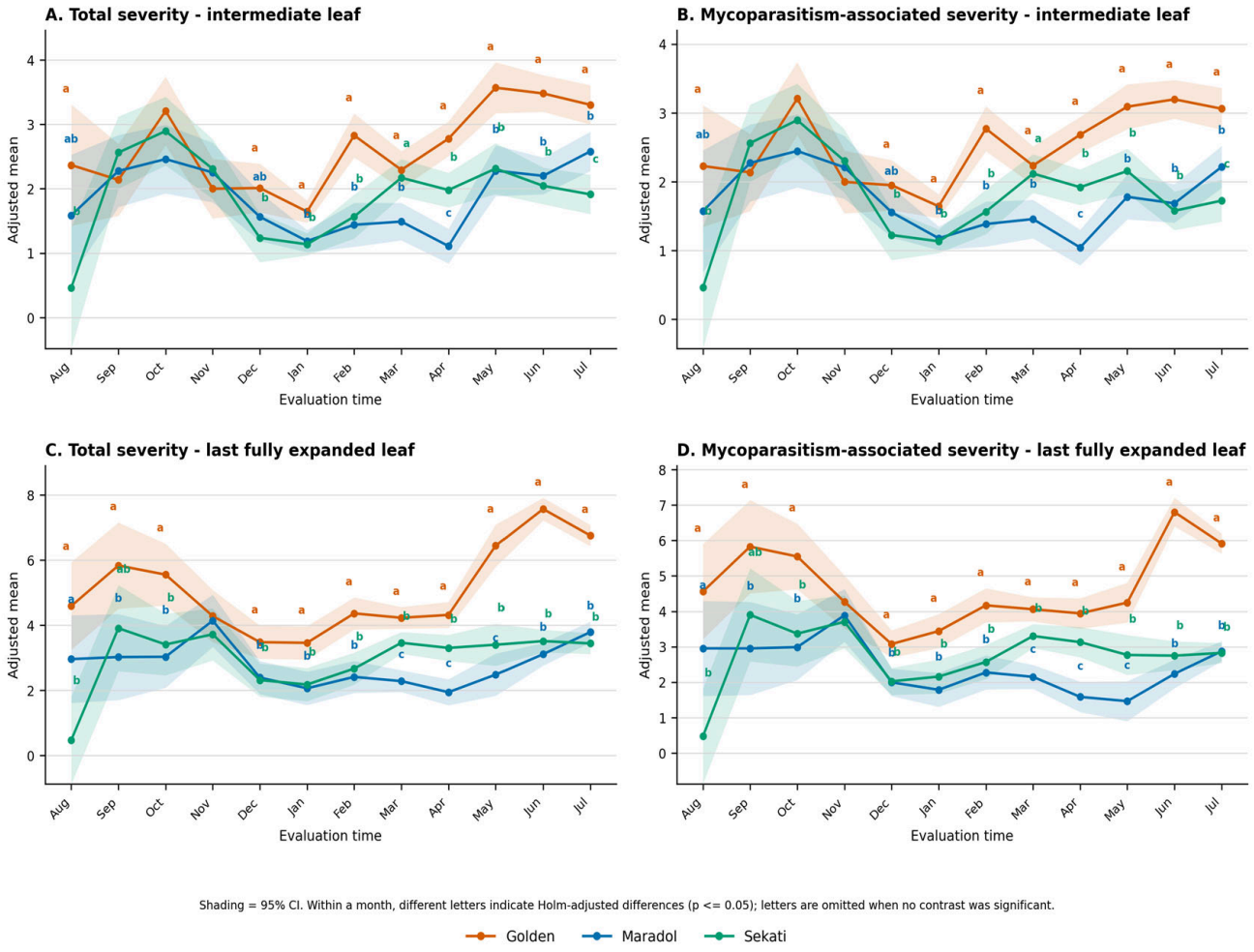

Figure 3. Model-adjusted monthly means of total papaya black spot severity and mycoparasitism-associated black spot severity on the intermediate leaf [BSS-IL (**A**) and BSSm-IL (**B**)] and on the last fully expanded leaf [BSS-LFE (**C**) and BSSm-LFE (**D**)] for 'Golden', 'Maradol', and 'Sekati'. Shaded areas represent 95% confidence intervals. Within each month, different letters indicate significant differences among genotypes based on Holm-adjusted pairwise contrasts ($p \leq 0.05$); letters are omitted when no significant genotype contrast was detected. Means and confidence intervals are shown on the original scale.

The clearer discrimination among genotypes based on severity than on incidence is consistent with previous studies showing quantitative variation in papaya resistance to black spot [2–4]. Maradol has previously been identified among genotypes with lower disease intensity, whereas Golden has generally shown greater susceptibility [3]. Severity may therefore be more informative than incidence for distinguishing partial resistance

when most plants or leaves become infected but differ in the amount of affected tissue. This is also consistent with the use of severity-based measurements for characterization of varietal resistance in this pathosystem [17].

The strongest genotype differences occurred during periods of greater disease intensity, suggesting that resistance differences became more evident as disease pressure increased. Temporal variation in black spot severity has also been associated with seasonal changes in environmental conditions, particularly rainfall and temperature [12,23].

Mycoparasitism-associated severity broadly followed the temporal pattern of total black spot severity. One plausible explanation is that increasing disease severity provided a greater amount of lesion tissue available for colonization by fungi associated with *A. caricae*. Previous studies have shown that *H. pulvinata* and *Acremonium*-like fungi can colonize black spot lesions, with substantial variation in colonization among fungal isolates [10,11]. Nevertheless, the parallel increase in BSS and BSSm does not demonstrate disease suppression. Because total severity and mycoparasitism-associated severity were analyzed as separate responses, the present results indicate temporal association rather than a causal reduction in disease attributable to fungal colonization.

3.6. Proportion of Non-Colonized Lesions and Temporal Occurrence of Morphological Fungal Groups

The proportion of non-colonized black spot lesions was affected by genotype, evaluation time, and their interaction on both leaf positions. On the intermediate leaf (PNCL-IL), significant genotype contrasts occurred in January, May, and June 2017. On the last fully expanded leaf (PNCL-LFE), significant contrasts occurred in November 2016 and in January and April through July 2017. Adjusted PNCL values were close to zero during several early assessments and increased during later evaluations, particularly on the last fully expanded leaf (Figure 4). Because PNCL was calculated as $100 \times (BSS - BSSm)/BSS$, higher values indicate a greater relative proportion of black spot severity without visible signs of fungal colonization, whereas lower values indicate a greater relative contribution of lesions showing signs of mycoparasitism. Because leaf positions were analyzed separately, apparent differences between positions should be regarded as descriptive rather than formal comparisons.

The low PNCL values observed during several early assessments indicate that a substantial proportion of the diseased area was associated with lesions showing signs of fungal colonization. Conversely, the increase in PNCL during later evaluations suggests that this relationship changed over time, with a larger fraction of total black spot severity occurring without visible mycoparasitic colonization. Such temporal variation is consistent with previous reports showing considerable variability in the ability of fungi associated with *A. caricae* lesions to colonize black spot lesions [10,11]. In controlled experiments, isolates of *H. pulvinata* differed markedly in their capacity to colonize lesions, indicating that mycoparasitic activity is not necessarily uniform among fungal genotypes or over time [10].

The occurrence of structures morphologically consistent with *H. pulvinata* varied with evaluation time ($p < 0.0001$) and showed a genotype × evaluation-time interaction ($p = 0.0002$), although the overall genotype effect was not significant ($p = 0.2938$). Significant monthly contrasts occurred in November 2016 and April, June, and July 2017. The occurrence of *Acremonium*-like fungi was affected by genotype ($p = 0.0204$), evaluation time ($p < 0.0001$), and their interaction ($p = 0.0050$), with significant genotype contrasts in December 2016 and April, June, and July 2017. Other fungal morphotypes also varied over time ($p = 0.0010$) and showed a genotype × evaluation-time interaction ($p = 0.0004$), although their overall genotype effect was not significant ($p = 0.5097$); the only significant monthly contrast occurred between Golden and Maradol in April 2017 (Figure 5A–C).

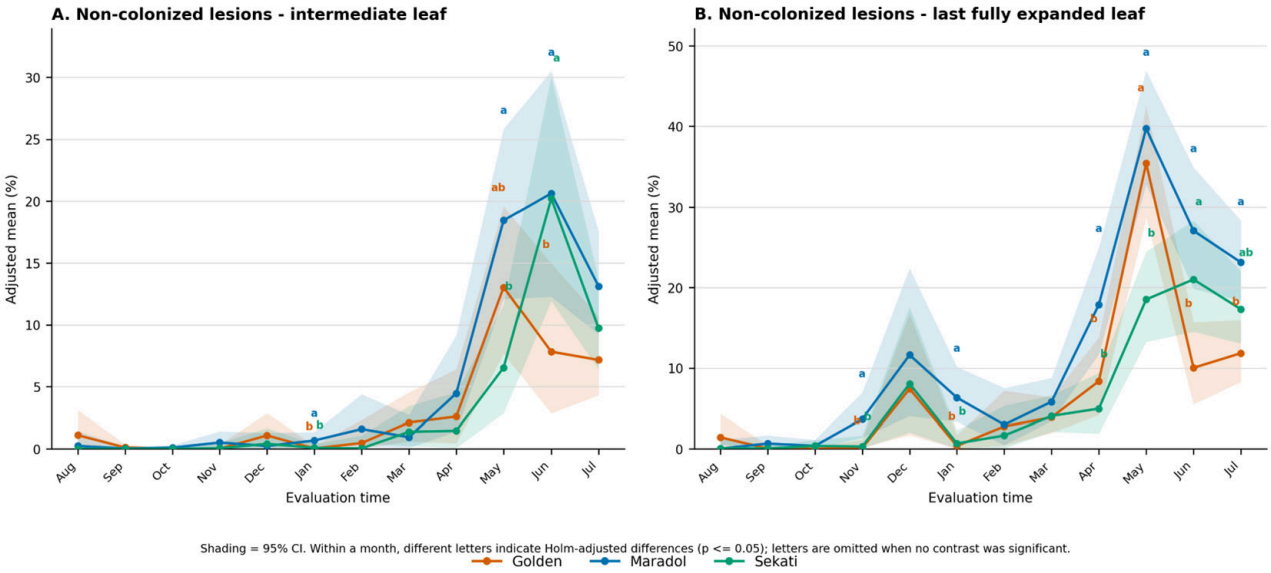

Figure 4. Model-adjusted monthly means of the proportion of non-colonized black spot lesions on the intermediate leaf (PNCL-IL; (**A**)) and on the last fully expanded leaf (PNCL-LFE; (**B**)) for 'Golden', 'Maradol', and 'Sekati'. PNCL was calculated from total black spot severity and mycoparasitism-associated severity as $100 \times (\text{BSS} - \text{BSSm})/\text{BSS}$. Higher values indicate a greater relative proportion of black spot severity without visible signs of fungal colonization. Shaded areas represent 95% confidence intervals. Within each month, different letters indicate significant differences among genotypes based on Holm-adjusted pairwise contrasts ($p \leq 0.05$); letters are omitted when no significant genotype contrast was detected. Means and confidence intervals are shown on the back-transformed percentage scale.

Structures morphologically consistent with *H. pulvinata* and *Acremonium*-like fungi were the predominant fungal groups detected on black spot lesions, whereas other morphotypes occurred less frequently. Previous studies have likewise recovered *H. pulvinata* and *Acremonium* spp. from *A. caricae* lesions and demonstrated their capacity to colonize diseased papaya tissue [10,11]. In addition, controlled studies have shown different temperature optima for growth and sporulation of these fungal groups, with *H. pulvinata* performing best around 21 °C and *Acremonium* spp. approximately between 20 and 25 °C [15]. These differences provide a biologically plausible context for their distinct temporal profiles in the field, although they do not establish that temperature was the cause of the observed seasonal changes.

The temporal variation observed here therefore suggests that the natural occurrence of fungi associated with black spot lesions is dynamic and may depend on the simultaneous availability of host lesions, fungal biological characteristics, and environmental conditions. The establishment and activity of microbial antagonists under field conditions are known to depend on their ecological fitness and their ability to tolerate local biotic and abiotic conditions [26]. However, because temperature, relative humidity, rainfall, lesion availability, and fungal abundance were not modeled jointly, the observed patterns should not be interpreted as evidence of physiological adaptation or causal climatic effects.

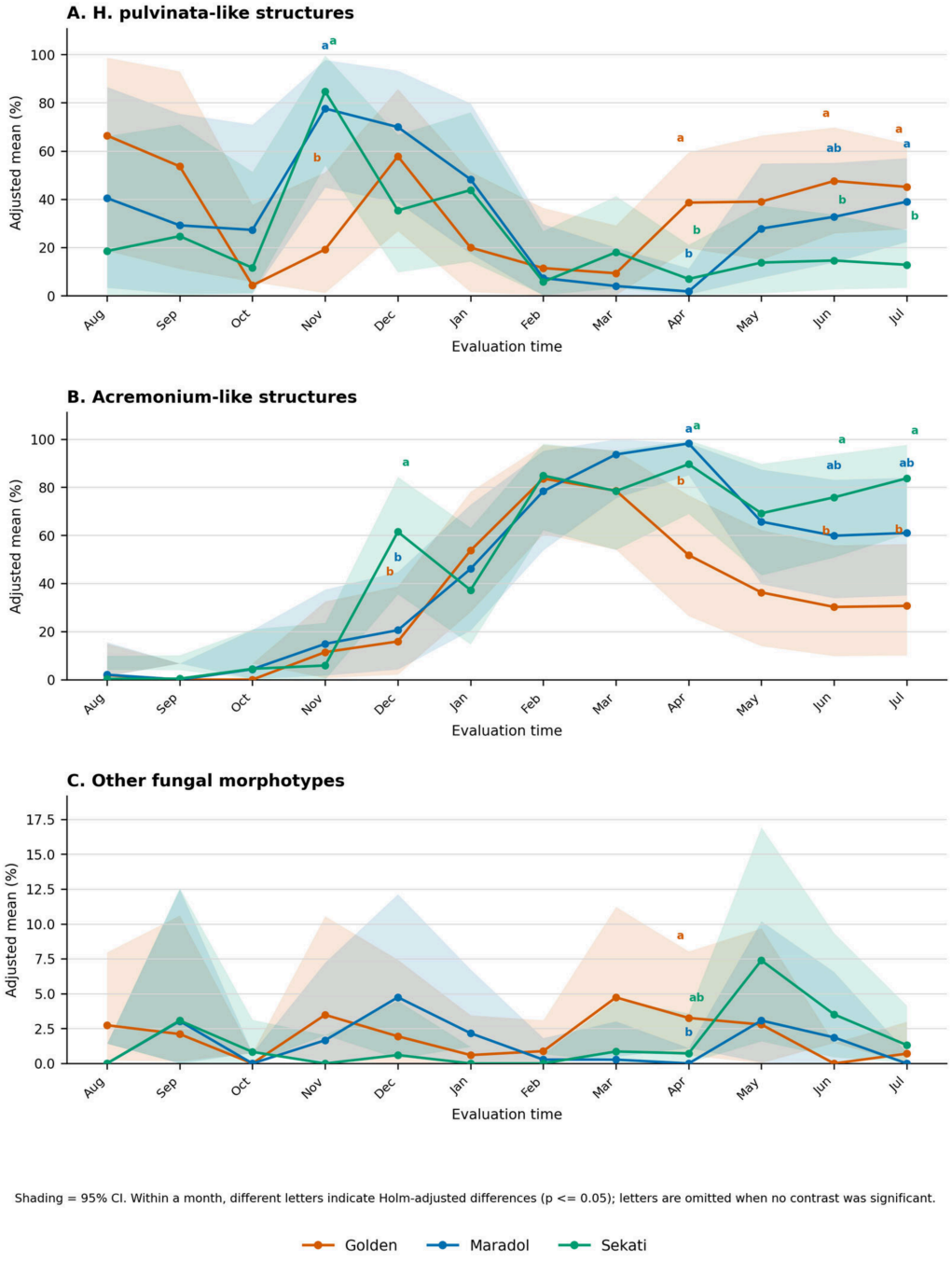

Figure 5. Model-adjusted monthly occurrence frequencies of structures morphologically consistent with *H. pulvinata* (**A**), *Acremonium*-like fungi (**B**), and other fungal morphotypes (**C**) for 'Golden', 'Maradol', and 'Sekati'. Shaded areas represent 95% confidence intervals. Within each month, different letters indicate significant differences among genotypes based on Holm-adjusted pairwise contrasts ($p \leq 0.05$); letters are omitted when no significant genotype contrast was detected. Means and confidence intervals are shown on the back-transformed percentage scale; the analyses used the original, non-normalized occurrence frequencies.

3.7. Implications for Biological Control and Study Limitations

The repeated natural occurrence of fungal structures morphologically consistent with *H. pulvinata* and *Acremonium*-like fungi indicates that papaya black spot lesions can be naturally colonized by fungal groups previously investigated as potential antagonists of *A. caricae* [10,11]. Their marked temporal variation and significant genotype × evaluation-time

interactions further indicate that fungal occurrence was not determined by host genotype alone and did not remain constant throughout the production cycle.

These observations support further investigation of *H. pulvinata* and *Acremonium*-like fungi as candidates for biological control. Previous studies have demonstrated their ability to colonize *A. caricae* lesions and have provided experimental evidence of antagonistic or mycoparasitic potential [10,11]. However, the present study evaluated natural fungal occurrence rather than experimentally imposed biocontrol treatments. Consequently, the observed association between fungal colonization and black spot lesions does not demonstrate reductions in pathogen sporulation, secondary inoculum, disease progress, or yield loss. The transition from the identification of potential antagonists to their practical use requires controlled efficacy experiments, assessment of environmental persistence, formulation development, application-method validation, and biosafety evaluation [27]. Controlled inoculation experiments are therefore required to quantify these effects and establish biological-control efficacy.

The absence of preserved cultures and molecular data represents an additional taxonomic limitation. Future studies should combine morphological characterization with fungal isolation and molecular identification and should evaluate pathogen–mycoparasite interactions under controlled environmental conditions. Because papaya genotypes differ in quantitative resistance to black spot [2,3], another relevant step would be to determine whether mycoparasitic fungi interact consistently with host resistance. If antagonistic efficacy is confirmed, combining partially resistant genotypes with compatible fungal antagonists could be investigated as a component of integrated black spot management. The development of microbial agents for sustainable disease management also requires appropriate strain selection, mass production, formulation, application technology, and validation under representative field conditions [28]. At present, however, this possibility should be regarded as a research prospect rather than a management recommendation derived directly from the present observational study.

4. Conclusions

Papaya black spot and the natural occurrence of fungi associated with its lesions showed pronounced temporal variation during the 12-month evaluation period. Significant genotype × evaluation-time interactions for all response variables demonstrated that differences among Golden, Maradol, and Sekati depended on the evaluation month. Disease severity discriminated genotypes more consistently than incidence, with Golden generally showing greater severity than Maradol and Sekati, particularly on the last fully expanded leaf.

The proportion of non-colonized lesions also varied among genotypes and over time, indicating temporal changes in the relative contribution of lesions showing signs of fungal colonization. Structures morphologically consistent with *H. pulvinata* and *Acremonium*-like fungi were repeatedly detected on black spot lesions and exhibited distinct temporal occurrence patterns.

Because fungal identification was based exclusively on reproductive morphology and the study evaluated naturally occurring colonization, these findings neither constitute molecular confirmation of fungal identity nor demonstrate biological-control efficacy. Nevertheless, the repeated occurrence of these morphologically defined fungal groups supports their further investigation as potential antagonists of *A. caricae*. Future studies should combine culture-based and molecular identification with controlled experiments designed to quantify effects on pathogen sporulation and disease progress and to evaluate their compatibility with partially resistant papaya genotypes and other components of integrated disease management.

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Abbreviations

The following abbreviations are used in this manuscript:

BSI	papaya black spot incidence
MLI	incidence of leaves containing mycoparasitized lesions
BSS-IL	total black spot severity on the intermediate leaf
BSSm-IL	mycoparasitism-associated black spot severity on the intermediate leaf
PNCL-IL	proportion of non-colonized lesions on the intermediate leaf
BSS-LFE	total black spot severity on the last fully expanded leaf
BSSm-LFE	mycoparasitism-associated black spot severity on the last fully expanded leaf
PNCL-LFE	proportion of non-colonized lesions on the last fully expanded leaf
IND	independent residual covariance structure
AR(1)	homogeneous first-order autoregressive residual covariance structure
ARH(1)	heterogeneous first-order autoregressive residual covariance structure

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