Stomatal Penetration and Temporal Dynamics of Ingress of Two Fungal Isolates Associated with Leaf Spot Disease of JASMINE (*Jasminum sambac* L.)

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Abstract:*Jasmine (Jasminum sambac L.) is an ornamental crop grown in South and Southeast Asia for its flowers for garlands, tea and essential oil production. However, certain fungal foliar and floral diseases significantly reduce its yield. In this study, Colletotrichum sp.-like (cylindrical conidia) and Fusarium sp.-like (lunate conidia) isolates causing leaf spot disease were characterized based on the ingression process and fungal germination in planta. Four-day single-spore cultures in PDA of isolating C1 (Colletotrichum sp.-like) and F2 (Fusarium sp.-like) were obtained. Suspensions of 10^8 conidia mL^{-1} were made and sprayed onto young leaves of jasmine. Specimen collection was done at 2, 6, 12, 24, 36, 48, 60 and 72 h after inoculation (hai). Formalin-acetic acid was used to clear the tissues and fungal structures are selectively stained using lactophenol-acid fuschin. Average ingression sites (IS) were counted for each time point under 50X magnification, then IS per leaf area (cm^2) was calculated. Fungal isolates remain at the surface of the leaf until 36 h. IS of C1 and F2 almost doubled at 48 hai. However, ingression sites declined at 72 hai for both pathogens, which either imply a progression of sub surface colonization or unsuccessful penetration. Leaf yellowing and a few spots were observed at 48 hai for F2 and at 60 hai for C1. More severe necrotic leaf spots with yellow halo (severity rating of 5) were seen in plants inoculated with Fusarium sp.-like isolated than in those inoculated with Colletotrichum sp.-like isolated. Lastly, at 48 to 60 hai, it was evident that the pathogen started to seek for stomata, which seemed to be the preferred penetration site for both fungal pathogens.*
Key words: *Colletotrichum sp., Fusarium sp., Jasminum sambac L., leaf spot, Sampaguita*

INTRODUCTION

Through the course of evolution and constant parasitic association with a plant host, pathogenic fungi have developed elaborate mechanisms for tissue penetration or ingress. Less evolved pathogens, those with a less elaborate mechanism for plant infection, perform ingress by just taking advantage of the openings of the preformed mechanical wounds on the host surface. On the other hand, more evolved pathogens, those with a more elaborate mechanism for infection, have developed more sophisticated artillery for ingress by seeking for natural openings such as the stomata or hydathodes. Some pathogens even enter through the specialized natural openings such as nectar holes, in the case of *Erwinia amylovora* that enters through apple tree's nectarthodes. Moreover, some fungi also form specialized structures that are assisted by cell wall degrading enzymes in order to penetrate the host effectively. These specialized structures include the appressoria and penetration peg. Lastly, effective fungal ingress is necessary in order to obtain nutrients from the host for the parasite's growth and reproduction (Abawi and Lorbeer, 1971; Spencer, 1973).

Fusarium spp. and *Colletotrichum* spp. are both conidiogenous fungi that developed elaborate mechanisms of ingress to their respective hosts. These mechanisms include the formation of cytoplasmic bridges or connections with the host cell, which do not only permit nutrient sequestration but can perform organelle exchange (Hoch, 1977; Hoch and Fuller 1977). The formation of germ tubes and penetrative hyphae is another effective mechanism for ingress, especially for the tactile search of suitable sites of penetration. Germ tubes and hyphae can directly grow into the epidermal layer after enzymatic degradation of plant cuticular layer. Germ tubes can also enter the host's natural openings such as the stomata. Finally, the formation of an enlarged hyphal tip called the appressorium is another structure that many parasitic fungi

produce to assist ingress. Due to the expanded surface area, fungal adhesion is also aided by the appressorium. The appressorium can either be simple or compound (single branch or contiguous). Contiguous appressorium can perform multiple ingress events. Initial events leading to host penetration involves the formation of the penetration peg, which is also the primary hyphal penetration event (Gold, R.E. and K. Mendgen, 1984).

Jasmine (*Jasminum sambac* L.), locally known as Sampaguita, is an ornamental crop grown mainly in South and Southeast Asia for its flowers for garlands, tea production, and essential oil extraction. In addition, jasmine flower colors other than white are also desired to be bred, thus giving this national flower added value and to make the flowers bigger in size (Magdalita et al., 2013). However, its potential for pharmaceutical, cosmetic and beverage production is not yet fully explored in the Philippines. In the Philippine archipelago, jasmine cultivation is only localized in Luzon, specifically in the provinces of Laguna, Quezon, and Pampanga (Rimando, 2003). In these areas, only the flower buds are harvested and sold. During peak production months of March to July, Pampanga produced and sold the most number of buds, with an average of 97,600 floral buds in 2009. On the other hand, Lucena in Quezon and Sta. Cruz in Laguna produced the least number of floral buds with at least 2,825 and 1,330 in 2009, respectively (Sanchez, 2011). The decrease in yield during the peak months in these areas is attributed mostly to severe weather conditions, insect pests and diseases. Major insect pests include whiteflies (*Dialeurodes kirkaldyi* Kotinsky), micro-Lepidoptera (tiny moth) and Thrips (Order *Thysanoptera*). These insects are most likely vectors of pathogens that aids in the spread of disease in the field. Moreover, diseases like root rot, leaf yellowing, sclerotium wilt, leaf curling, foliar and floral diseases (Sanchez et al., 2010; Balidion et al., pers. communication) significantly reduces the yield in the past years.

The flower buds of jasmine are extremely sensitive to high moisture and low temperature. Exposure to such conditions can lead to browning or rotting of the foliar and floral parts. Fungal outgrowths are favored during these warm, humid conditions and are commonly observed in aborted buds and crop residues. Fluctuating weather during summer months make the diseases more severe as it leads to the proliferation of both fungal and bacterial pathogens, as well as saprophytic microbes. Furthermore, improper disposal of the pruned plant materials and unsynchronized management strategies with neighboring farms further aggravates the situation in growing areas resulting to a disease-conducive environment.

An investigation of the leaf spot disease was done. It was found out that the illness was caused by either a *Colletotrichum* sp.-like or *Fusarium* sp.-like fungus or both. In order to characterize this pathosystem, an ingress study was conducted which primarily aimed to: (i) describe the ingression process and fungal germination of *Colletotrichum* sp.-like (cylindrical conidia), and *Fusarium* sp.-like (lunate conidia) isolates in leaves of jasmine and its symptom development; and (ii) determine the average putative infection sites in inoculated leaves for each fungal isolate per unit area (cm^2) at each time point for a 72-hour observation period.

MATERIALS AND METHODS

Isolation and pathogenicity testing of fungal isolates.

Tissue plating technique was used to isolate fungal pathogens associated with the leaf spot disease of jasmine. Tissue sections (approximately 2 mm X 2 mm) obtained from the margin of leaf spot and healthy tissue portion were incubated in Potato Dextrose Agar (PDA) plates for two days at room temperature. After incubation, actively growing mycelial plugs (0.8 cm in diameter) were obtained using a cork borer and transferred to a fresh PDA plates. The fungal cultures were allowed to grow for seven days until sporulation occurred. Spore suspensions of 10^8 conidia mL^{-1} were prepared and inoculated onto healthy jasmine plants to test its pathogenicity or whether the same set of symptoms can be observed. Incubation of plants lasted for 7 to 14 d until leaf spot symptoms were observed. Fungal isolates that tested positive for the pathogenicity test were used in the subsequent ingress experiment.

A pure culture of the fungal isolates and preparation of plant materials.

Stored fungal isolates (at 4°C) tagged as C1 (*Colletotrichum* sp.-like) and F2 (*Fusarium* sp.-like) were revived and grown in (PDA) for four to five days. These fungal isolates were already previously tested positive for pathogenicity in foliar parts of jasmine. Afterwards, the isolates were subjected to continuous white light for three days in order to induce spore production. Single conidial cultures of the isolates were obtained following the procedure by Goh (1999). Spore suspension from a 10^{-6} dilution series of fungal colonies was spread plated onto PDA plates. Single conidial colonies in PDA were grown for two to three days. Mycelial discs (0.8 cm diameter) of the pure cultures were transferred to PDA slants and incubated at room temperature for four days prior to inoculation. Moreover, stem cuttings of mature jasmine plants were grown for at least three months. Lastly, jasmine plants were treated with fungicide every week, or as needed, in order to eliminate contaminating fungal pathogens and to maintain healthy plants prior to inoculation.

Inoculation of fungal isolates in Jasmine foliar parts for ingress experiment.

The fresh inoculum was prepared from the fungal isolates in the form of conidial suspensions by flooding 7 day-old slant cultures with 5 mL sterile distilled water and lightly scraping the colony with a sterile glass rod or teasing needle. The suspensions were filtered through sterilized cheesecloth to separate mycelial fragments from the conidia. The suspensions were standardized up to 10^{8} conidia mL^{-1} using a hemacytometer. Conidial suspensions were mixed with Tween-20 to make 1%, v/v solution (i.e. 1 mL for 100 mL suspension). Tween-20 served as the surfactant and to prevent washing off of the inoculum. Young jasmine plants, approximately three months old were used as the test specimens. A total of 16 test plants were used, *i.e.*, two plants per time point were used to serve as test plants for each of the two fungal isolate. Using a clean atomizer, the inoculum suspension was sprayed onto the surface of the plants. Inoculation was done late in the afternoon to prevent heat stress to the plant as well as desiccation of the fungal spores. The inoculated leaves were covered with a clear polyethylene bag for 24 h to avoid cross-contamination and to maintain high relative humidity to favor pathogen growth. The test plants were placed under greenhouse conditions for 72 h for specimen sampling.

Microscopic observation of the ingression process.

Specimen collection was done at 2, 6, 12, 24, 36, 48, 60 and 72 h after inoculation in the form of tissue sections. Ten sample tissue sections of about 0.25 cm^2 (0.5 cm X 0.5 cm) was obtained per time period per fungal isolate, for a total of 160 samples for the entire experiment. The tissue samples were placed in a glass vial containing 5 mL of a fixing-clearing solution. Formalin-acetic acid (Rawlin's FAA) fixing and pigment clearing solution containing 50% ethanol, formalin, and glacial acetic acid (10:1:1 v/v/v) were used to make the sampled tissues translucent suitable for microscopic examination (SC-APS, 1967). This fixing-clearing solution contains ethanol in low concentration thus will slowly decolorize plant pigments including white pigments (mainly anthoxanthins) in flower petals (Gardner, 1975). All tissues were soaked for 24 h or until the tissue becomes translucent. Replacement of the solution is necessary if decolorization did not occur after 24 h.

After decolorization, the tissues were immersed in 50% lactic acid for 5 min to clear it further (Boke, 1968). Cleared tissue specimens were stained using lactophenol-acid fuchsin for 15 min and dipped in lactophenol to remove excess stain. Tissues were mounted on a glass slide in a small drop of 50% glycerine solution or with the fuchsin stain. Average ingression sites (IS) were counted per isolate per leaf area (cm^2) for each time period.

Symptoms were observed in every time period, and photographs of the germinated fungal conidia were taken. Quantitative severity rating for the leaf spot symptom is as follows: 0 (no symptoms), 3 (5% of leaf area with leaf spot), 5 (10% of leaf area with leaf spot), 7 (25% of leaf area with leaf spot), 9 (50% of leaf area with leaf spot) (Table 4). The devised disease severity rating mentioned implies that the leaf spots do not cover the majority of the leaf area with necrotic spots.

Statistical Design and Analysis

The evaluation of the ingression sites (IS) for each time point followed the Completely Randomized Design (CRD) with ten replications per time period. That is, ten tissue samples were obtained for each time point per fungal isolate for a total of 160 samples. All data gathered were subjected to one-way Analysis of Variance (ANOVA) at $\alpha = 0.05$. Groupings of statistically similar means followed the Duncan's Multipple Range Test (DMRT, at $\alpha = 0.05$). All computations and statistical analysis were aided by the Statistical Analysis System (SAS) software v1.4 (SAS Institute, Inc., Cary, NC, USA, 1976).

RESULTS AND DISCUSSION

Characteristic symptoms and microscopic examination of fungal isolates.

Characteristic symptoms of the leaf spot disease were characterized for both fungal pathogens (Figure 1). Isolate F2 (*Fusarium* sp.-like) produces larger necrotic spots which later coalesce to form blight-like symptoms. On the other hand, C1 (*Colletotrichum* sp.-like) produces necrotic lesions with a characteristic yellow halo.

Microscopic observations of the pathogenic isolates using a light microscope (OPTIKA™, OPB-192, Hicksville, New York, USA, 2006 model) revealed distinct morphology and cultural characteristics (Figure 2). Moreover, dynamics of growth in PDA were also noted. Distinct fungal growth and sporulating structures were also described. Comparison of cultural characteristics in PDA revealed a clear distinction between the two isolates (Table 1). In PDA, the 14-day old C1 fungal colony has a purple flat wholly growth while F2 has a pinkish irregular profuse growth. Microscopic examination of the pure cultures of the pathogenic isolates revealed its gross morphology in PDA (Table 2). Isolate F2 has lunate, septate conidia, which is a typical characteristic of the *Fusarium* species. On the other hand, isolate C1 has cylindrical septate conidia, which is a typical characteristic of the *Colletotrichum* species. A study by Wikee et al. (2011) identified *Colletotrichum* species causing leaf spot in Jasmine plants in Vietnam. They described two new species as *C. jasminigenum* and *C. jasmine-sambac.* These species are morphologically and culturally distinct from the isolates identified in this study, thus may be another set of pathogens causing leaf spot in jasmine.

Microscopic examination of inoculated jasmine plants

Two hours after inoculation, observation of the possible ingression or penetration sites were done using a light microscope (Zeiss, Axiostar Plus™, Gottingen, Germany, 2001 model, 5x (FOV 20 mm)). Table 3 shows the average number of ingression sites (IS) observed. The number of ingression sites per square centimeter of the inoculated tissue was computed by dividing the average IS by the area of the field of view (FOV) of the scanning (5X) objective, (0.2 cm FOV radius at 5X objective magnification) which is equivalent to $(0.2\text{ cm})^2 \pi$ or 0.1256637061 cm^2.

Figure 3 and 4 shows the dynamics of the count of ingression sites observed for F2 and C1 fungal isolates, respectively, throughout the 72-hr observation period. Based on the figures, it can be inferred that the fungal isolates remained on the surface of the leaf until 36 h. At the peak of ingress count for both pathogens (48 hai), there was an abrupt increase in ingression sites. However, although more IS is observed in F2, the fold increase of IS from 2 hai to 48 hai is higher in C1 (2.6 fold increase) than in F2 (2.3 fold increase). This observation was also reported in other fungal pathosystems where an increase in penetration and initial appearance of symptoms is observed at 48 h after inoculation (Miles et al., 2009 and Mandy, et al., 2009). However, ingression sites seem to decline from 48 hai at 72 hai for both pathogens. During this period there was a 33% decrease in the IS of F2 while there was a 50% decrease in the IS of C1. This phenomenon might indicate that the pathogens have already penetrated inside the leaf and has established a network of hyphal growth. Another reason for such scenario is that a number of fungal conidia were not able to penetrate successfully due to the effect of host defenses. This phenomenon was also observed in the

ingress of *Colletotrichum* spp. in cowpea (Latunde-Dada, et al., 1999) and in the ingress of *Fusarium oxysporum* Fo47 in pea (Benhamou and Garand, 2001).

Figure 5 and 6 show representative tissue samples at 48 hai and 72 hai, respectively, which are evidently teeming with stained ingression sites (red or pink to deep purple). There is no significant difference in the density of ingression sites at 2 hai until 24 hai. Heavily stained red or pink to deep purple leaf samples can be observed at 48 hai (Figure 5), in which highest IS was also observed. In addition, even the decrease in the number of ingression sites from 48 hai to 72 hai was also evident for both fungal isolates in the stained tissues. Symptoms such as leaf yellowing were just observed at 48 hai for F2 and at 60 hai for C1. More severe symptoms were observed (Table 5) in plants inoculated with the *Fusarium* sp.-like fungal isolate (severity rating of 5) than in the plants inoculated with the *Colletotrichum* sp.-like isolate (severity rating of 3). One explanation for such observation in C1 is that either there is a latency in the appearance of symptoms or the plant effectively counteracts the pathogen. Lastly, in most cases, fungal hyphal growth was observed to be subsurfacial for both fungal isolates. Subsurface colonization starts as early as 12 to 24 h for *Colletotrichum* spp. (Latunde-Dada, et al., 1999, Kumar, et al., 2001) and 6 to 12 h for *Fusarium* spp. (Kang and Buchenauer, 1999; Peraldi et al., 2011).

Fungal conidia for both isolates did not germinate significantly at 2 hai. Conidial germination started at around 6 hai and continued until 36 hai. At 48 hai, extensive networks of mycelial growth were observed profusely growing within the plant tissue. This was observed for both fungal isolates. At 48 to 60 hai, it was evident that the pathogen started to seek for stomatal openings. In most tissues, it was observed that the preferred penetration site of both fungal pathogens is the stomata, using its germ tube and hypha. The observed ingression sites that were stained red or pink to deep purple are sites of active fungal growth near or at the stomatal ridges or opening itself. Figure 7 shows the evident preferential ingression by F2 isolate and C1 isolate which is through the stomata (red arrow) at 72 hai. Isolate F2 was observed to seek for stomata as early as 48 to 60 hai. The fungal mycelia in the were also stained red or pink to deep purple.

Furthermore, there was no evident formation of appressoria in neither of the two pathogens. Appressoria usually forms from 6 to 24 hai as reported in previous studies (Kuo, 1999; Liao et al., 2012). Both pathogens are known to form appressorium *in vitro* under nutrient-depleted conditions (Manandhar, et al., 1995; Goswami and Kistler, 2004; Wikee et al., 2011 and Boenisch and Schafer, 2011). In most fungal plant pathogens, the appressorial formation is usually triggered by a decrease in nutrient supply or total nutrient depletion. The fungal isolates in this experiment were subjected to infection to their native host, thus supplying them with ample nutrients to persist and not form appressoria. Lastly, these specialized fungal structures are known to aid in mechanical penetration during infection (Bell and Wheeler, 1986 and Howard and Ferrari, 1989). Interestingly, fungal isolates in this experiment prefer penetration through the stomata and not through mechanical penetration.

SUMMARY AND CONCLUSION

A time-course experiment was conducted to examine and characterize the temporal ingression of two fungal isolates associated with the leaf spot disease of jasmine. Two fungi were isolated, a *Colletotrichum* sp.-like (cylindrical conidia) and *Fusarium* sp.-like (lunate conidia). Suspensions of 10^8 conidia mL^{-1}, from the 7-day old cultures, were prepared and sprayed onto the surface of leaves of three-month old jasmine plants. Sample tissues (0.5 cm X 0.5 cm) were collected at 2, 6, 12, 24, 36, 48, 60 and 72 hours after inoculation (hai)). Formalin-acetic acid (FAA) was used to fix and clear the tissue samples. Fixed and cleared tissues were stained using lactophenol-acid fuschin. Average ingression sites (IS) were counted for each time period and the average IS per leaf area (cm^2) observable under the scanning objective (50X magnification) was also calculated.

Fungal isolates remained on the surface of the leaf until 36 h. Fungal structures such as mycelia progressed in the sub surface afterward. IS of C1 and F2 peaked at 48 hai. The fold increase of IS from 2 hai

to 48 hai is higher in F2 (2.6 fold increase) than in C1 (2.3 fold increase), although more ingression sites are observed in F2. The abrupt increase in IS for both fungal pathogens are accompanied by the appearance of the leaf spots, with more severe symptoms observed in F2 (severeity rating of 5). However, ingression sites significantly declined at from 48 to 72 hai for both pathogens. There was an observed 50% decrease in IS of C1, while 33% decrease for F2. This observation indicates successful subsurface colonization of the fungal pathogens because they are no longer visible on the surface. More leaf spots were observed in plants inoculated with the *Fusarium* sp.-like fungal isolate than in those inoculated with *Colletotrichum* sp.-like isolate. This is despite higher ingression sites observed in C1. Furthermore, at 48 and 60 hai, it was evident that the pathogens started to seek for stomata, which is the preferred penetration site for both isolates. No formation of appressoria was observed in neither pathogens *in planta*, which strengthens the evidence of a preference for fungal penetration via the stomata and not through mechanical penetration.

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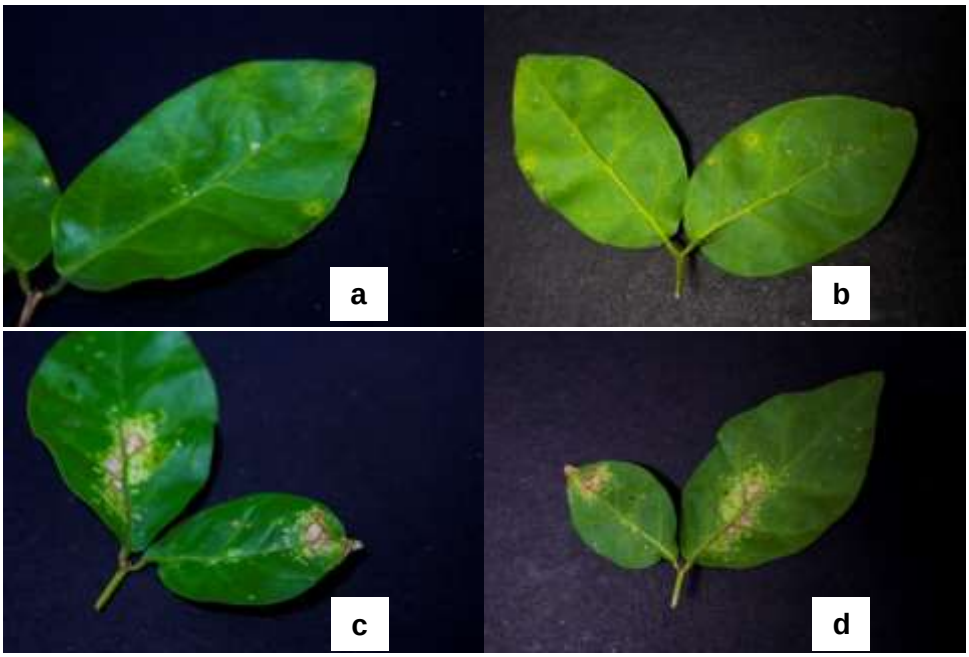
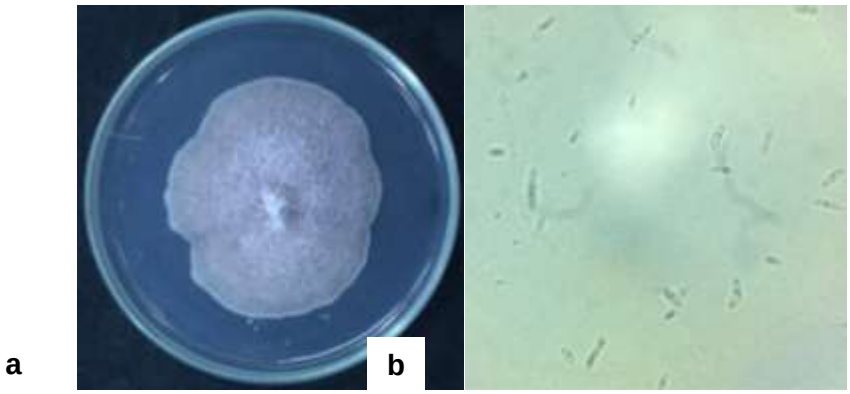


Figure 1. The characteristic symptom of the leaf spot disease as observed in jasmine plants. Characteristic symptom caused by the infection of the *Colletotrichum* sp.-like isolate (an adaxial view; b, abaxial view) and those of the *Fusariuum* sp.-like isolate (c, adaxial view; d, abaxial view).



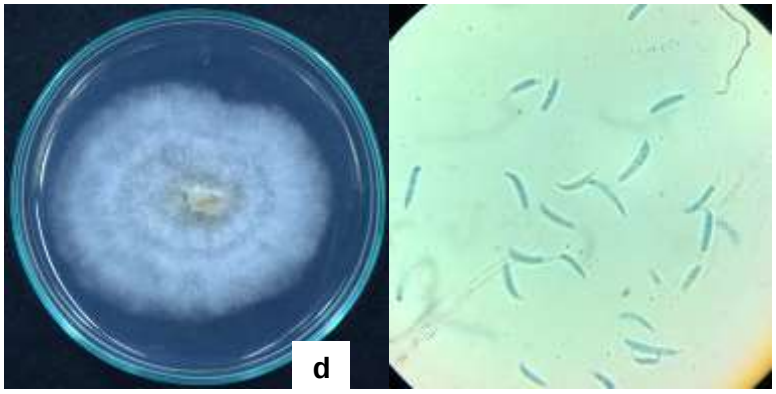


Figure 2. The growth of the fungal isolates in PDA after 14 d and their characteristic gross morphology. *Colletotrichum* sp.-like isolate in PDA (a) and its characteristic cylindrical conidia (b). *Fusarium* sp.-like isolate in PDA (c) and its characteristic lunate conidia.

Table 1. Comparison of cultural characteristics of the fungal isolates in PDA (14-day old) associated with the leaf spot disease of jasmine.

Cultural Characteristic	F2 (*Fusarium* sp.-like)	C1 (*Colletotrichum* sp.-like)
Color	7 d: white to light pink 14 d: pink	7 d: white to light purple 14 d: dark purple
Form	irregular, slightly filamentous	circular, slightly irregular
Elevation	raised	flat
Edges	curled	entire
Pigmentation on media	presence, light pink	presence, purple
zonations	presence	absence

Table 2. Comparison of gross morphology of fungal isolates associated with the leaf spot disease of jasmine, grown in PDA for 14 d.

Gross Morphology, Specialized Structures	F2 (*Fusarium* sp.-like)	C1 (*Colletotrichum* sp.-like)
Vegetative hyphae	hyaline, branched, septate	hyaline, branched, septate
Conidiophores	hyaline, unbranched, septate	hyaline, unbranched, septate
Coidia	lunate with multiple septations (2-4)	cylindrical with single septatation
Chlamydospores	absence	absence
Setae	absence	presence

Table 3. Estimated number of ingression sites per square centimeter of the inoculated area across the 72 h observation time.

Hours after inoculation (hai)	Ingression sites per cm^2 ± s.d. [1]	
	F2	C1
2	151 ± 14	133 ± 5
6	167 ± 35	146 ± 28
12	183 ± 8	202 ± 36
24	186 ± 12	199 ± 8
36	215 ± 35	321 ± 53
48	347 ± 44	350 ± 68
60	324 ± 9	324 ± 9
72	233 ± 47	175 ± 24

[1]computed as (= average no. of ingression sites / 0.1256637061 cm^2)

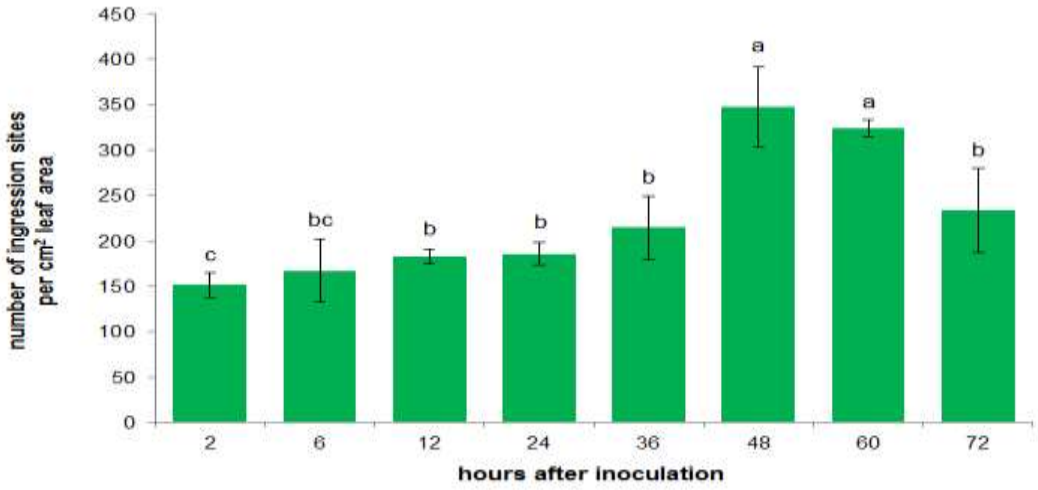


Figure 3. An average number of ingression sites per square centimeter of leaf area in jasmine leaves inoculated with C1 (*Fusarium* sp.-like) isolate. Note: Bars refer to the standard error of the samples. Data parameter with the same letter grouping were determined by ANOVA, DMRT at α=0.05, and are not significantly different.

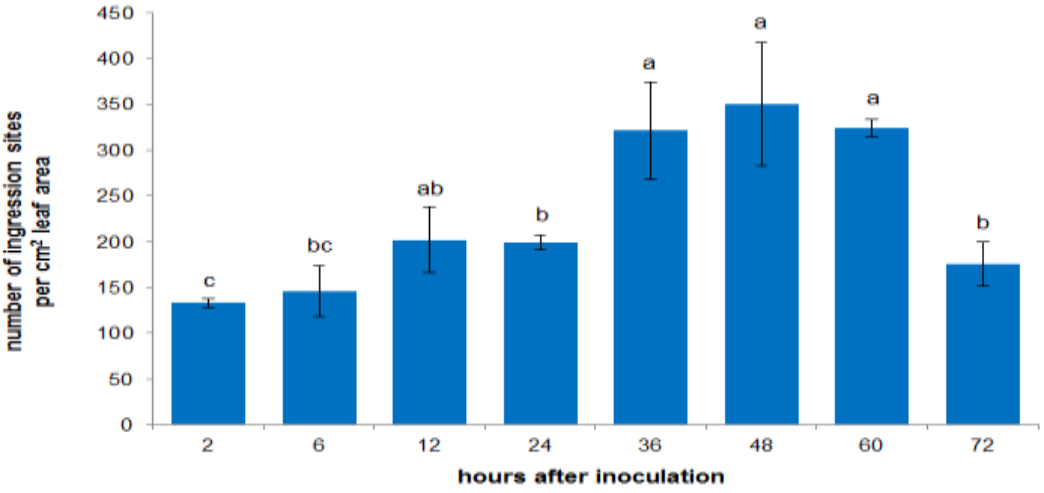


Figure 4. An average number of ingression sites per square centimeter of leaf area in jasmine leaves inoculated with F2 (*Colletotrichum* sp.-like) isolate. Note: Bars refer to the standard error of the

samples. Data parameter with the same letter grouping were determined by ANOVA, DMRT at α=0.05, and are not significantly different.

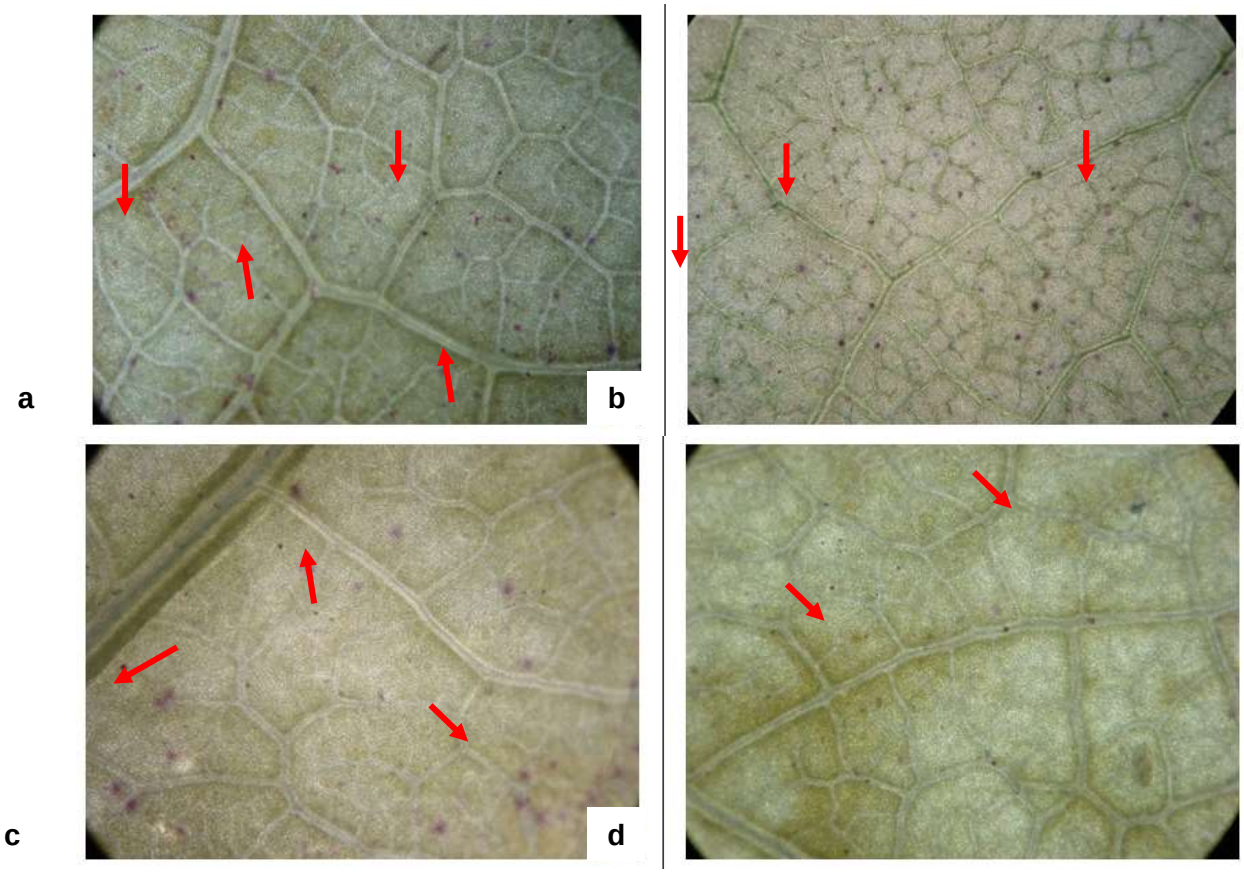


Figure 5. Photomicrographs (50X magnification) of the possible ingression sites (stained red/pink) as observed in the inoculated leaves at 72 hai (a, b) and 48 hai (c, d) for each fungal isolate: (a, c) F2 and (b, d) C1.

Table 4. Disease severity rating scale devised to numerically characterize the leaf spot symptom observed in inoculated jasmine plants.

Disease Severity Rating	Symptoms Observed
0	no symptoms
3	5% leaf area with necrotic spots
5	10% leaf area with necrotic spots
7	25% leaf area with necrotic spots
9	50% leaf area with necrotic spots

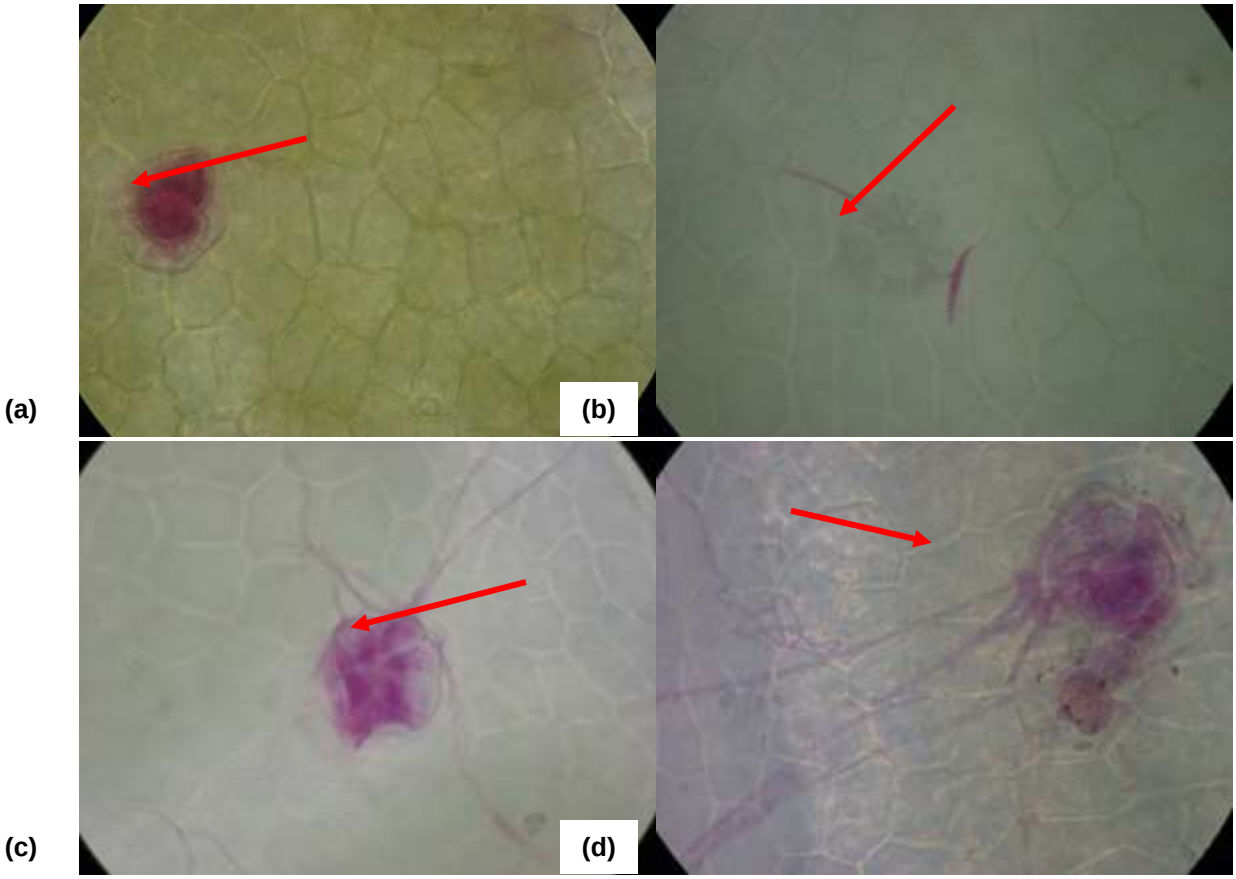


Figure 6. Microscopic evidence (400X magnification) that the preferential ingression by C1 (a) and F2 (b, c, d) is the stomatal opening of jasmine leaf, using only its germ tube and hypha as ingression structures. Tissue samples were obtained at 72 hai (a, b), at 48 hai (c) and at (d) 60 hai.

Table 5. Disease severity rating of inoculated jasmine plants throughout the 72-hour observation period.

Hours after inoculation (hai)	Disease Severity Rating	
	F2	C1
2	0	0
6	0	0
12	0	0
24	0	0
36	0	0
48	3	0
60	3	3
72	5	3