https://doi.org/10.17221/82/2020-HORTSCI

Pathogenic and biological characterisation of T-DNA insertional mutants of a *Colletotrichum gloeosporioides* casual organism of apple anthracnose

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Citation: Azeem M., Zhou Z.S., Zhang J.X., Khaskheli I.M., Rui Z.J., Khaskheli A.J., Ali S. (2021)**:** Pathogenic and biological characterisation of T-DNA insertional mutants of a *Colletotrichum gloeosporioides* casual organism of apple anthracnose. Hort. Sci. (Prague), 48: 51–62.

Abstract: Anthracnose leaf spot caused by *Colletotrichum gloeosporioides* is an important disease of apples, resulting in serious damage to the fruit production. In this paper, the pathogenic and physiological characters of sixteen isolates and the wild isolate "Stj16" were studied. In the current study, we generated *C. gloeosporioides* strains expressing green fluorescence by introducing a GFP gene via an Agrobacterium tumefaciens-mediated transformation (ATMT). To confirm the subcellular localisation of the A2799 gene, an *A2799gfp* fusion expression mutant was constructed. After observation of the fusion expression, the *A2799gfp* fusion expression protein was located in the peroxisomes of the cell. The pathogenicity results showed that the mutants A4204, M44, A1919, A3638 and A1598 lost the pathogenic capability and virulence, however, the virulence of the mutants A1764, A439, A3885, G1183, A3144, A1649 and A2675 increased significantly to the apple fruits compared to the wild isolate "Stj16". The biological study indicated that a Rose Bengal Agar (RBA) medium decreased the mycelium growth, but it can increase the sporulation for most of the isolates. The mutant A4204 does not grow well at pH 4.0 and pH 8.0, and mutant M44 just has the optimum growth at pH 8.0, and a 12 h light and 12 h dark condition stimulates the sporulation for most of the tested mutants, but the A1764 mutant more sporulated at regular dark conditions. All the mutants and "Stj16" grew vigorously at 25 °C–30 °C, for "Stj16", it produced the highest number of conidia at 30 °C compared with the other temperatures. Based on the biological study, we found the best growing and sporulation conditions for all the tested isolates. The information generated in the present study will facilitate molecular research on this devastating fungus.

Keywords: leaf spot; subcellular gene location; virulence, sporulation; optimum conditions

Anthracnose is one of the important diseases of tropical, subtropical and temperate fruits caused by the *Colletotrichum gloeosporioides* pathogen; it causes serious damage to the fruits and considerable economic losses in various crops worldwide (Amelie Grammen 2019). Anthracnose leaf spot is a severe apple disease caused by *C. gloeosporioides* in China, its infection initially manifests as round to elliptic

Supported and funded by " National Key Research and Development Project No. 2016YFD0201100.

https://doi.org/10.17221/82/2020-HORTSCI

shaped and water-soaked spots on the leaves and sunken spots on the fruit that finally lead to the early and fast leaf falling and fruit rot. The disease prefers humid, warm conditions and disperses by rain splash and moist winds (Martinez-Culebras et al. 2000, 2003; Sanders, Korsten 2003; Xiao et al. 2004). Under an affirmative environment, the disease can result in 75% defoliation, weakening the plants and reducing the yield (Gonzalez et al. 2006; Bogo et al. 2012).

Generally, Anthracnose has varied infective strategies and an intricate epidemiology, possess a pathogenic and non-pathogenic manner on the target and alternate host (Munda 2014; Wenneker et al. 2015; De Silva et al. 2017). Anthracnose is instigated by fungi that produce germ tubes and form a melanised appressorium, which assist its penetration into the host's epidermal cells (Michielse et al. 2005; Kenny et al. 2012). The physiological components such as the pH, temperature, light intensity and relative humidity have been described to have a profound influence on the pathogenicity of various filamentous fungi. The optimal temperature for the maximum hyphal growth of *Colletotrichum spp* was frequently found between 25 °C and 30 °C. The high temperature may weaken the fungal growth, and in some cases, mortality of the pathogen can occur (Moriwaki et al. 2003).

A series of biological studies on other filamentous fungi have been performed, for example, V-8 agar, oat meal agar and potato carrot agar media allowed good mycelial growth, but not an optimal one for strawberry anthracnose, potato dextrose agar (PDA) and chloramphenicol (CM) initiated optimum development and good sporulation of all the isolates (Es-Soufi et al. 2018), the maximum hyphal growth and sporulation of *C. gloeosporioides* was observed in a PDA culture media for some other fungi (Fernando et al. 2000; Stanly et al. 2013; De Costa, Chandima 2014; Es-Soufi et al. 2018). The overall aim of this study was to assess the behaviour of sixteen insertion mutants and the wild isolate "Stj16" of *C. gloeosporioides* associated with apple anthracnose leaf spot by altering the cultural traits, media composition, temperature fluctuation, pH and light regimes on the mycelial progress and conidia production. Meanwhile, the pathogenicity and phenotype characteristics were also studied.

MATERIALS AND METHODS

Mutants of *C. gloeosporioides*. Sixteen isolates of the *C. gloeosporioides* insertion mutants' library (A4204C1, A2675, A2799, A1643, A439, A2983, A1649, A1598, A3036, A1919, A3638, A3144, A3885, A1764, G1183, M44) and the wild type "Stj16", stored in the laboratory of molecular plant pathology, Institute of Pomology, CAAS, which were originally obtained from apple anthracnose disease were used in the study.

Subcellular localisation of A2799. To determine the subcellular localisation of the A2799 gene, the pGapneor44-A2799gfp plasmid was constructed and confirmed through a polymerase chain reaction (PCR) analysis. The fusion constructs were transformed into the wild isolate "Stj16" and the obtained overexpression isolate. The mycelia, conidia of the overexpression isolate were observed under a Leica DM5000B microscope (Germany).

Pathogenicity study of the mutants. To detect their pathogenicity, the healthy apple fruit's surface was sterilised with alcohol, punctured with a sterilised toothpick and a mini species of the hyphae of the mutants was inserted into the wound, then incubated in incubator at 28 °C, the symptom development was observed and recorded every day.

Phenotype observation of the mutants. The fungal vegetative growth was assessed by measuring the colony diameter on PDA plates after incubation for 8 days at 26 °C. The conidia numbers were calculated using a haemocytometer, the appressoria formation was observed under electron microscope.

Germination of the conidia and appressorium formation. The conidial germination and appressorium formation were observed on a hydrophobic glass slide. The conidial suspensions were adjusted to a density of 10^4/mL, then a concentrated droplet was dropped onto the centre of the glass slide placed in the (12%) water agar plates. The plates were incubated at 28 ℃ in the dark, the conidial germination rate was evaluated at 12 h post induction under the microscope.

Biological and physiological studies. Evaluation of the colony expansion and sporulation of the insertion isolates were carried out to find the variation among the insertion mutants under the composition of the different culture media, pH, light intensity and temperature fluctuation, ensuring the best condition to further study their pathogenic mechanism.

Effects of culture media composition. Ten different culture media, i.e., potato dextrose agar (PDA, potato 200 g, dextrose 20 g, agar 20 g, distilled sterilised water 1 000 mL); oat meal agar (OM, rolled oat 60 g, agar 20 g, distilled sterilised water

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1 000 mL); complete agar medium (CM, $(Ca(NO3)_2$ $4H_2O$, 1.5 g; KCl, 0.5 g; KH_2PO_4, 0.03 g; $MgSO_4$ $7H_2O$, 0.5 g; tryptone, 1 g; glucose, 10 g; yeast extract, 1 g; and agar 15 g in 1 L distilled water, pH 7.0); corn meal agar (corn meal 50 g; agar 15 g; dextrose 10 g; distilled sterilised water 1 000 ml); potato carrot agar (PCA, potato 20 g, carrot 20 g, agar 20 g, distilled sterilised water 1 000 mL); V-8 juice agar (tomato 174 mL, carrot 8 mL, onion 4 mL, "brinjal" 4 mL, spinach 4 mL, lettuce 4 mL, lady finger 4 mL, agar 15 g, $CaCO_3$ 2 g, distilled water 800 mL); yeast dextrose agar (YDA, yeast extract powder 10 g, dextrose 10 g, agar 18 g, distilled water 1 000 mL); onion dextrose agar (OA, onion 200 g, agar 18 g, 1 000 mL distilled water); water agar (WA, 18 g agar melted in 1 000 ml distilled autoclaved/sterilised water); Rose Bengal agar (RBA, rose bengal powder 32 g, 1 L distilled water) were prepared according to their recipes. After preparation, all the media were sterilised in the autoclave. The sterilised medium (17 mL) was poured into each petri plate and amended with an antibiotic (cm 200 mg/mL) to avoid bacterial contamination. After solidifying of the medium, a 5 mm diameter hypha disk from an 8–10 day-old culture was cut by using a sterile cork borer and placed in the centre of the plate and then incubated at 26 ± 2 °C. The data of the colony growth diameter of each insertional mutant were recorded in millimetres on a daily basis until the plates were filled in each treatment.

Effect of the light intensity. The effect of the light on the growth of the insertion mutants of the *C. gloeosporioides* pathogen was studied by exposing the inoculated culture to alternate 24-hour dark, 24-hour light, 12-hour dark and 12-hour light cycles, three repetitions of each treatment were maintained. The inoculated plates were kept in an incubator at 26 ± 2 ℃ and the florescent light intensity was adjusted to the required level. The mycelia growth was recorded on a daily basis until the plates were filled with the mycelia growth in each treatment after the inoculation.

Evaluation of the different pH levels. The pH levels of the PDA medium were adjusted through a digital pH meter using 0.1 NaOH to increase the pH and 0.1 NHCL to decrease the pH. The sterilised media (17 mL) was poured into the sterilised petri plates and allowed to solidify. A 5 mm disc of the fungal culture was cut by using a sterile cork borer and placed in the centre of the PDA petri plates. The inoculated plates were incubated at 26 ± 2 °C for further growth for 7 days. The data of the colony diameter was recorded in mm, the conidia production was determined with three replications for each treatment through the aid of a haemocytometer using light microscopy.

Temperature fluctuations. To find the optimal temperature for the mycelia growth and conidia production of the insertion mutants of *C. gloeosporioides,* seven temperature stages (5 ℃, 10 ℃, 15 ℃, 20 ℃, 25 ℃, 30 ℃ and 35 ℃) were evaluated. For this study, a 5 mm mycelium disc of each insertion mutant was placed in the centre of the PDA petri dishes containing the corresponding antibiotics (200 mg/mL), then the plates were incubated in incubators under completely dark conditions.

Quantification of conidia yield of insertion mutants. To determine the conidia concentration of the insertion mutants of *C. gloeosporioides* in the different cultivations, the conidia have been harvested directly from the surface of the sporulating cultures by adding 10 mL of the sterilised water and gently rubbing the ten-day-old culture of insertion mutants of *C. gloeosporioides*. Two layers of cheese cloth were used to filter the conidial suspension eliminating the debris and mycelium. The volume of the conidia-bearing suspension used for counting was 10 µL conidia (1×10^6) though a haemocytometer using light microscopy. The sporulation was graded according to the number of conidia as: – = no sporulation, + = poor sporulation (0–50 conidia), +++ = good sporulation (50–100 conidia) and ++++ = sporadic sporulation (>150 conidia) per microscopic field.

Statistical analysis. The mutant's development data obtained from the trial were statistically analysed using an analysis of variance (ANOVA) performed by the DPS statistical analysis software (Tang, Zhang 2013).

RESULTS

Subcellular location of A2799. To identify the location of the targeted protein, a fluorescent reporter gene *A2799gfp* was cloned and inserted into the pGapneor44 plasmid and checked through a PCR analysis as shown in (Figure 1), then transformed into the wild isolate "Stj16", the subcellular location of *A2799gfp* was analysed through the observation of the green fluorescent protein (GFP) with fluorescent microscopy, it was present in the peroxisomes of the conidial cell (Figure 2).

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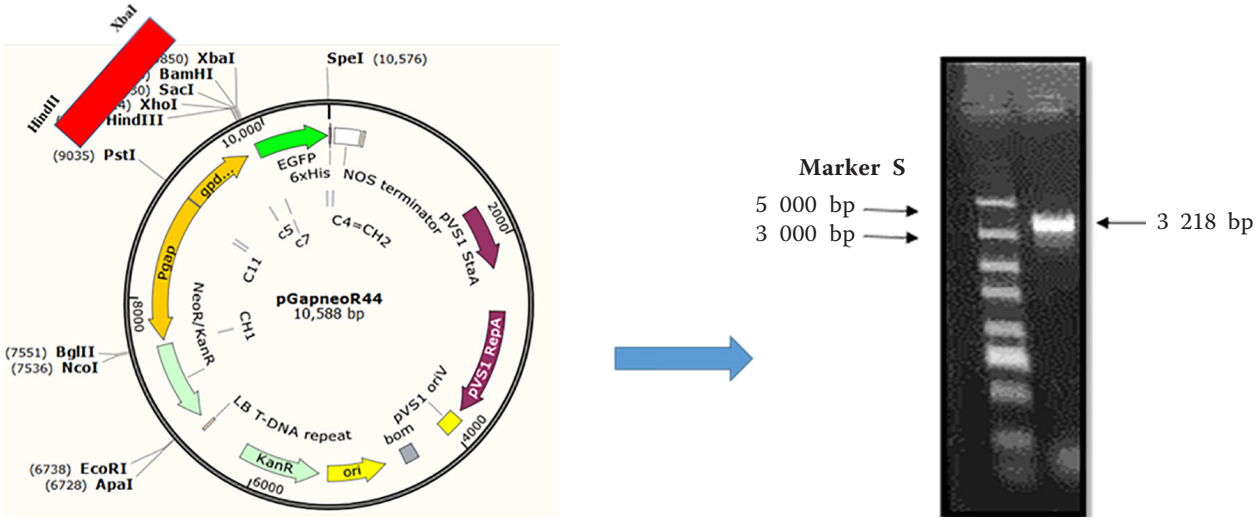

Figure 1. Expression vector pGapneor44 construction of the Gene-A2799GFP and PCR confirmation of the constructed plasmid

Identification of pathogenicity associated mutants. The pathogenicity test results showed that the wild-type "Stj16" and the insertional mutants G1183, A1649, A2675, A3144, A3885, A1764, A1643, A2983, A2799, A3036 produced distinct lesions on the apple fruits. However, the mutants A4204, A3638, M44, A1598 and A1919 entirely failed to generate necrotic symptoms and were not able to grow necrotrophically on the artificially wounded apple fruits. The wild-type "Stj16", G1183, A1649, A2675, A3144, A3885 and A1764 mutants induced extensive lesion formations on the epidermis of the fruits, when a hyphal pellet (1-mm-diameter) was spotted onto the surface of the fruit cuticle by being poked into an approximate 0.3 cm-deep hole by a toothpick. It indicates that the mutants are very aggressively virulent towards the host (Figure 3).

Phenotypic characterisation of the different isolates of *C. gloeosporioides*. In order to investigate the phenotypic characteristics of the different isolates, the morphological appearances of the insertional mutants of *C. gloeosporioides* on the PDA medium were studied, whereas the A1643, A2983, A439, A1919, A1598, A1649, A3036, A3144, A3885, and A1764 isolates produced a similar phenotype with the wild type "Stj16" with blackish-green coloured colonies. M44 produced off-white coloured colonies with flat mycelial growth and the A4204C1 isolates produced flat, a light-orange coloured growth appearance. While A2799 produced a white raised fluffy appearance. The A3638 mutant yielded the least mycelial growth with an irregular margin, the rest of the insertional isolates had a flat margin. Significant differences were observed on the substrate colour. Four isolates A1919, A2983, A2675 and "Stj16" produced a grey-black colour, isolate A4204C1 recorded a dark-orange one (Figure 4). The results indicated that all the mutants of *C. gloeosporioides* formed hyaline cylindrical conidia.

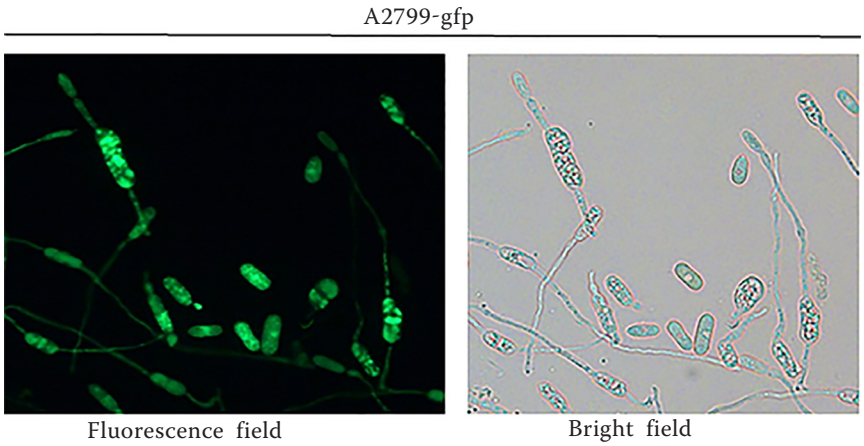

Figure 2. Expression of the GFP in the conidia and hyphae of the transformant A2799gfp

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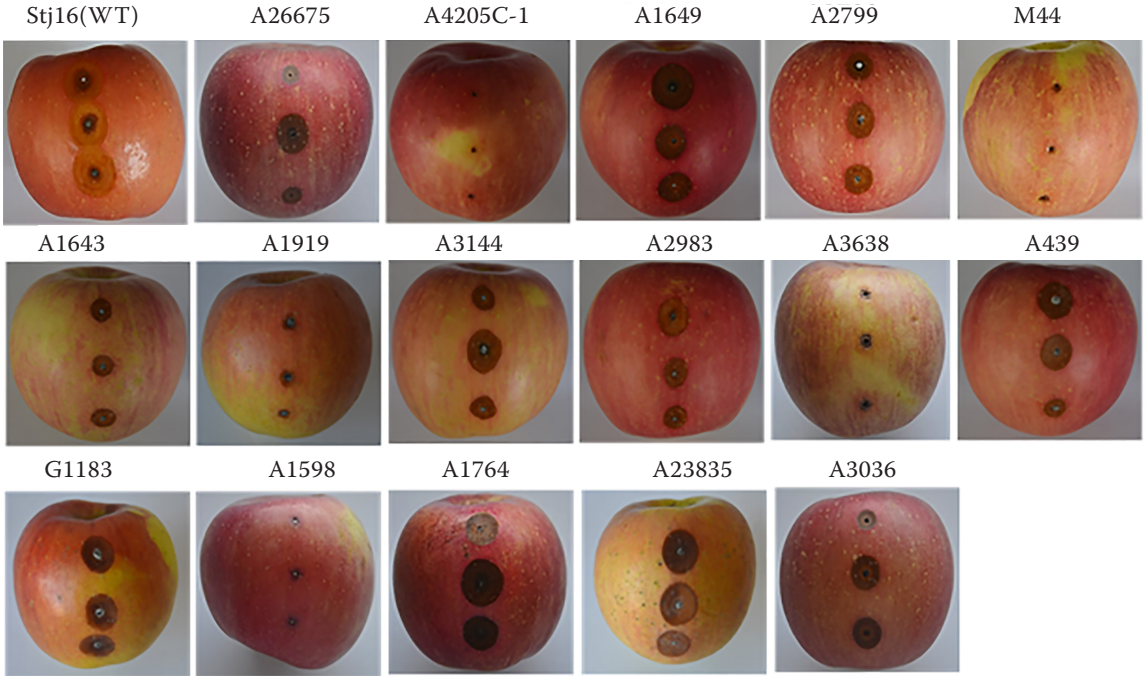

Figure 3. Development of the degree of pathogenicity for the insertional mutants on the apple fruits. The pathogenicity tests of the isolates were performed 8 days post inoculation at 26 °C

The data are the consequences of three different biological replicates

Germination of the conidia and appressorium formation. In view of this study, the appressorium formation plays a key role in the disease cycle of *C. gloeosporioides*. The conidia of the A2983, "Stj16" and A3638 mutants incubated on a hydrophobic glass slide surface exhibited unipolar conidia germination style. Whereas the conidia of the A3144, A2675, A3885, G1183 and A3036 mutants underwent bipolar germination and the appressorium appeared normal 12-h post inoculation. In addition, a mature appressorium formation was absent in M44, A439, A2799, A1643 and A4204 (Figure 4)

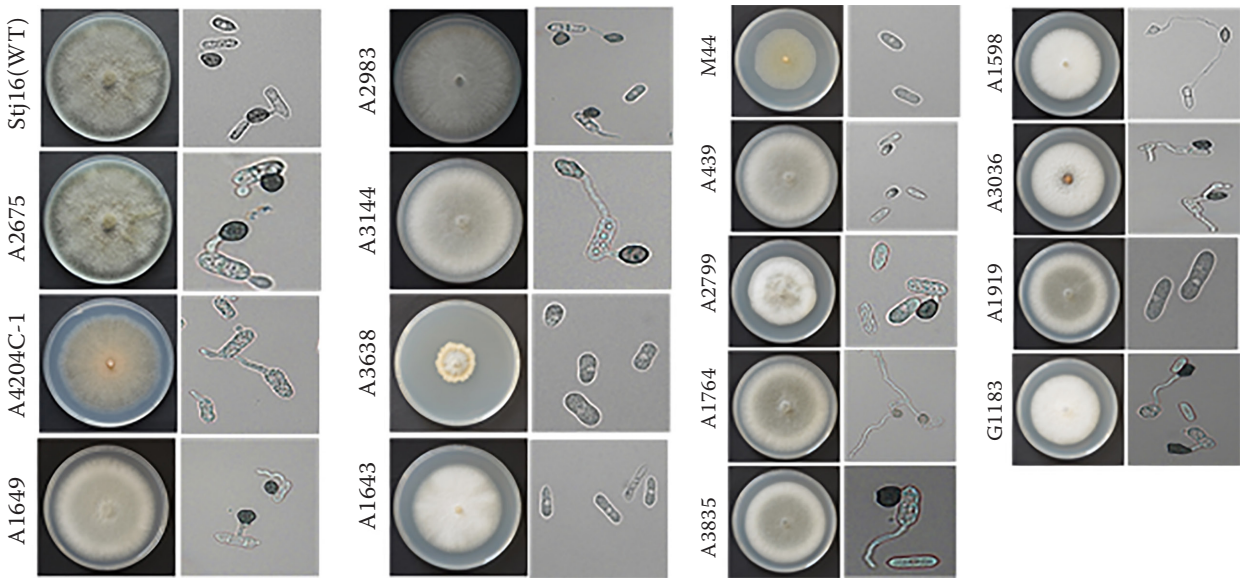

Figure 4. Colonies of the wild-type strain "Stj16" (WT), formed on the PDA incubation at 25°C in the dark. The photos were taken 6 days post-inoculation. The conidia and appressoria were examined on a hydrophobic glass slide using light microscopy after being cultured for 12 h

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Table 1. Effect of the different culture media on the vegetative growth of various isolates of *Colletotrichum gloeosporioides*

Isolates	PDA	PCA	V-8	Corn	CM	Oat meal	Onion	WA	YDA	RBA
Stj16	89.33^{a}	71.00^{a}	70.00^{fg}	75.00^{a}	88.66^{a}	71.00^{def}	71.00^{d}	50.00^{b}	88.00^{bc}	54.00^{a}
A1643	70.33^{d}	57.66^{h}	60.33^{k}	48.33^{j}	56.00^{j}	57.33j	61.00^{g}	18.00^{k}	55.66^{h}	20.66^{j}
A439	89.33^{a}	79.66^{b}	78.66^{b}	70.00^{b}	88.66^{a}	78.66^{b}	75.33^{b}	47.33^{c}	89.33^{a}	44.33^{c}
A1598	75.33^{c}	63.33^{f}	64.00^{j}	55.33^{h}	60.00^{h}	70.66^{ef}	59.66^{h}	28.33^{h}	65.00^{f}	22.33^{i}
A2983	88.66^{ab}	71.00^{e}	69.66^{gh}	56.33^{g}	74.66^{e}	70.33^{f}	74.00^{c}	26.00^{i}	75.00^{e}	44.00^{c}
A1649	88.66^{ab}	70.33^{e}	86.66^{a}	64.33^{d}	87.33^{b}	88.66^{a}	88.66^{a}	51.66^{a}	88.66^{ab}	35.00g
A4204	62.33^{e}	54.66^{i}	54.33^{l}	52.66^{i}	63.33^{g}	62.66^{h}	55.33^{j}	22.66^{j}	53.00^{j}	37.66^{f}
A3036	88.66^{ab}	70.66^{e}	68.66^{h}	62.33^{e}	77.33^{d}	76.33^{c}	70.33^{d}	35.66^{e}	79.66^{d}	34.33^{g}
A3885	88.66^{ab}	87.66^{a}	72.00^{e}	66.00^{c}	88.66^{a}	89.00^{a}	70.66^{d}	47.33^{c}	87.66^{c}	50.66^{b}
A2799	89.00^{ab}	62.33^{g}	73.66^{d}	55.33^{h}	70.00^{f}	72.33^{d}	68.33^{e}	42.00^{d}	88.66^{ab}	35.33^{g}
A3638	40.66^{f}	37.00^{j}	17.66^{n}	28.66^{l}	37.00^{k}	11.66^{k}	15.33^{k}	6.00^{l}	18.66^{k}	10.33^{k}
A2675	88.33^{b}	71.00^{e}	75.33^{c}	61.00^{f}	87.66^{b}	88.66^{a}	74.00^{c}	40.6^{d}	88.66^{ab}	50.00^{b}
A1919	89.33^{a}	75.00^{c}	74.33^{cd}	62.66^{e}	75.33^{e}	78.33^{b}	70.33^{d}	30.3^{fg}	74.66^{e}	34.66^{g}
A3144	89.33^{a}	70.66^{e}	71.00^{ef}	60.66^{f}	79.33^{c}	70.33^{f}	68.66^{e}	29.66^{gh}	75.00^{e}	39.33^{e}
A1764	88.66^{ab}	72.33^{d}	74.00^{d}	64.00^{d}	88.00^{ab}	72.00^{de}	70.33^{d}	31.33^{f}	88.33^{bc}	40.33^{e}
M44	75.00^{c}	58.00^{h}	65.66^{i}	39.33^{k}	60.00^{h}	67.00^{g}	64.66^{f}	7.00^{l}	62.33^{g}	41.66^{d}
G1183	62.33^{e}	54.33^{i}	43.66^{m}	53.00^{i}	57.3333^{i}	59.00^{i}	56.66^{i}	23.66^{j}	54.00^{i}	24.66^{h}

PDA – potato dextrose agar; PCA potato carrot agar; V-8 – juice agar; Corn – corn agar; CM – complete medium; Oat meal – oeat meal agar; Onion – onion dextrose agar; WA – water agar; YDA – yeast dextrose agar; RBA – Rose Bengal agar

compared to the wild-type strain. The mutants A1598 and A1764 generated an extended germ tube to form an appressorium, but the size of the appressorium was much smaller than the wild-type.

Assessment of growth characteristics on the different solid culture media. To further test how the fungal growth modified the medium on which they were cultured, ten various kind of solid media were evaluated against the growth characteristics of the *C. gloeosporioides* insertional mutants. The colony growth diameter and sporulation were considered as the studied factor. All the culture media facilitated the vegetative growth of the mutants with different means of development (Table 1). Among these culture media significantly allowed optimal colony growth of tested fungus A3036, A2675, A2799 and wild isolate "Stj16" was observed on potato dextrose agar and CM agar. The oat meal agar and potato carrot agar showed average mycelial growth, all the isolates produced the least vegetative growth on the rose bengal agar and water agar. The CM and rose bengal agar (RBA) stimulated the sporulation of the isolates A4204, A3036, A2675 followed by the PDA, PCA and V-8, while, the onion agar, and oat meal agar had low sporulation (Figure 5).

Influence of the light regimes stimulates the sporulation of the isolates. The response of the *C. gloeosporioides* anthracnose causing fungi investigation with regards to the duration of light exposure varies with the light intensity employed. The influence of the hyphae on the growth was often limited, but it had a bigger effect on the development of reproductive structures and the formation of spores (Figure 6). The light regimes' results showed that all the isolates grew well at an alternative 12 h dark and 12 h light cycle. The 24-hour dark cycle supported good growth of A2799, A1919 and the wild-type "Stj16", while the 24-hour light cycle stimulated the sporulation of A3036 and A4204 (Figure 7).

Increasing the pH level negatively affected the development of the isolates. The growth of *C. gloeosporioides* fungi is affected by the (pH) in a medium in which it gets nourishment, either by its effect on the availability of nutrients or directly by its action on the cell surfaces. All the isolates grew vigorously at all pH values (Figure 8). The optimum mycelial growth was at pH 6 and 7 and the optimum sporulation was calculated in a range from pH 5.0 to pH 7.0, the maximum number of spores were formed at pH of 6.0 and the lowest were formed at pH of 4.0 and pH 8.0, which indicates that a higher alkaline medium is not appropriate for the development of *C. gloeosporioides* (Figure 9).

Variation in temperature significantly affected the development of the isolates. To further under-

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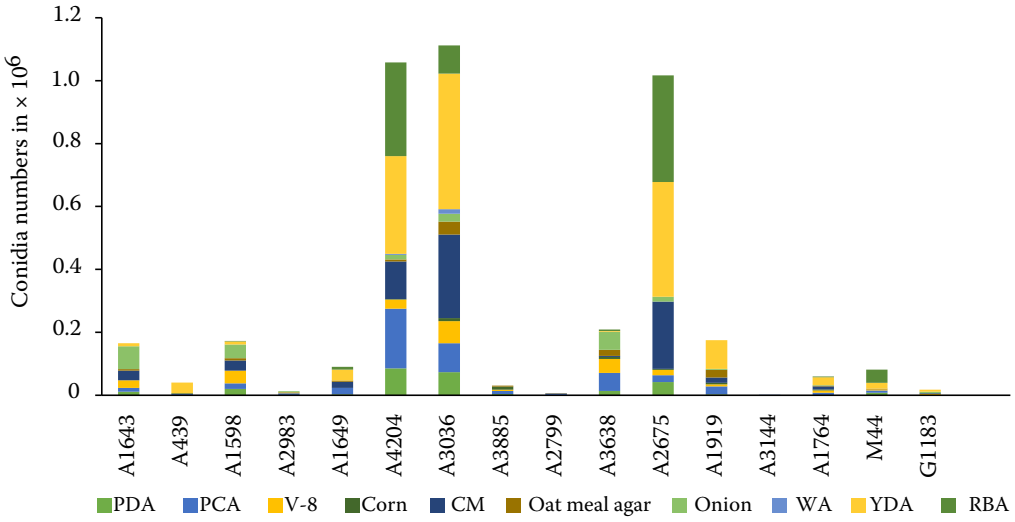

Figure 5. Effect of the different culture media on the sporulation of seventeen isolates of *Colletotrichum gloeosporioides*

The data are the average from three different biological replicates; for abbreviations see Table 1

stand the influenced mechanism of the *Colletotrichum* isolates, the effect of the different temperature levels on the growth of the *Colletotrichum* isolates was evaluated and recorded. The incubation temperatures remarkably influenced the colony growth of the test pathogen. The data showed that there was a significant difference found in the growth of the fungal isolates at the different temperature levels (Figure 10). The maximum growth in the *Colletotrichum* isolates was recorded at 25 ℃ and 30 °C. However, the growth of some insertional isolate's growth was noted as occurring moderately at 15 ℃, 20 ℃, and 35 ℃. All the isolates stopped their vegetative growth at 5 °C (Figure 11).

DISCUSSION

Colletotrichum gloeosporioides insertional mutants caused symptoms on the apple fruits related to the anthracnose. In our study, the pathogen was artificially inoculated onto the apple fruits, the symptoms produced by the virulent insertional mutants were similar to the symptoms observed under a natural infection. The symptoms appeared as necrosis; sunken lesions were distributed over the wounded outer part of the fruit. By comparing the pathogenicity of the insertional mutants with the wild type "Stj16" on the apple fruit, the infectious isolates A1764, A439, A3144, A1649, A2675 increased the infec-

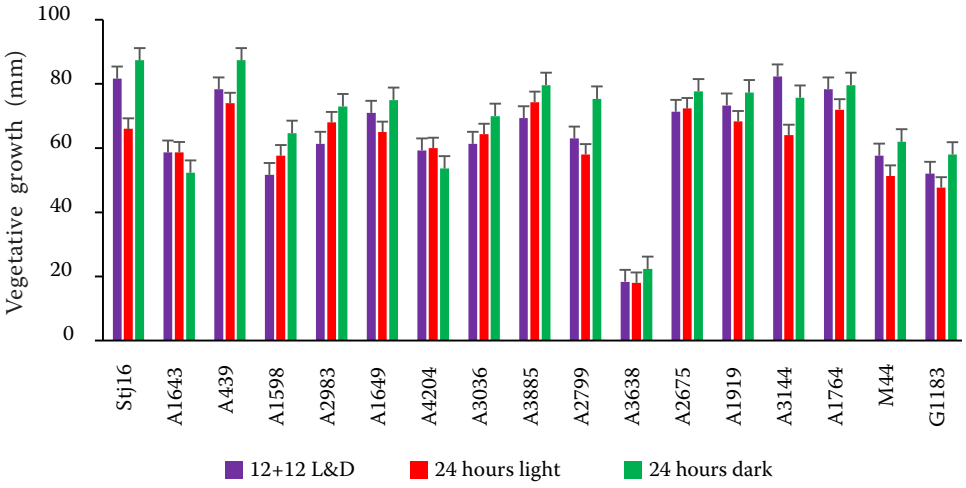

Figure 6. Effect of the light regimes on the vegetative growth of the insertional mutants in *Colletotrichum gloeosporioides*

The error bars represent the SD of the average from three different biological replicates

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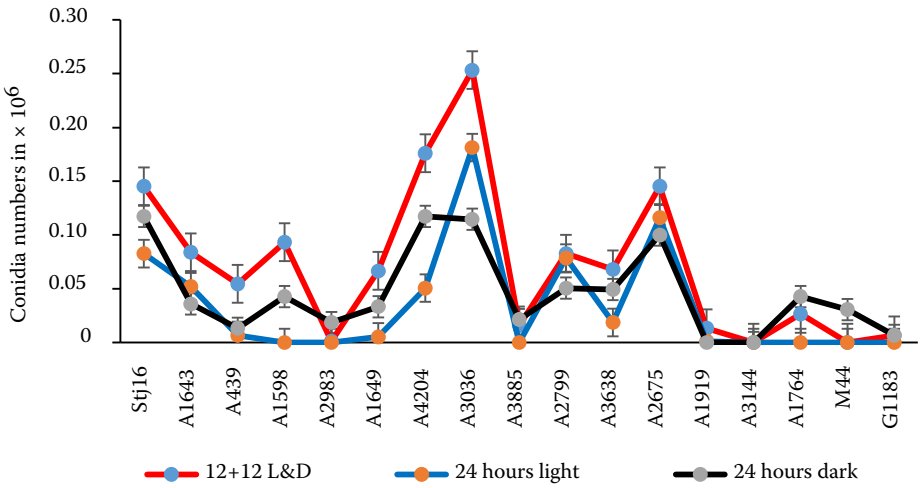

Figure 7. Influence of light regimes on the sporulation of T-DNA insertional mutants of the *Colletotrichum gloeosporioides*

The error bars represent the SD of the average from three different biological replicates

tion on the fruits, which represented a dominant level of aggressiveness among the studied mutants. A1919, A3036 induced less symptoms than A2675 and A2799 when inoculated into the wounded fruit, the rest of the mutants A4204, M44, A3638 and A1598 completely lost the ability of the pathogenicity compared to the corresponding wild type "Stj16". Hyde et al. (2009) explained in their research findings that the *Colletotrichum acutatum* isolate was able to infect the inoculated leaves.

Appropriate culture media and favourable environmental conditions resulted in the rapid growth and sporulation of various isolates. Fungi generally require a medium with different pH, light and favourable temperature conditions during the course of their development. These factors influence the stages of their life cycle. The development of *C. gloeosporioides* changed with the studied environmental factors, like temperature, pH, light and culture medium composition. In the current study,

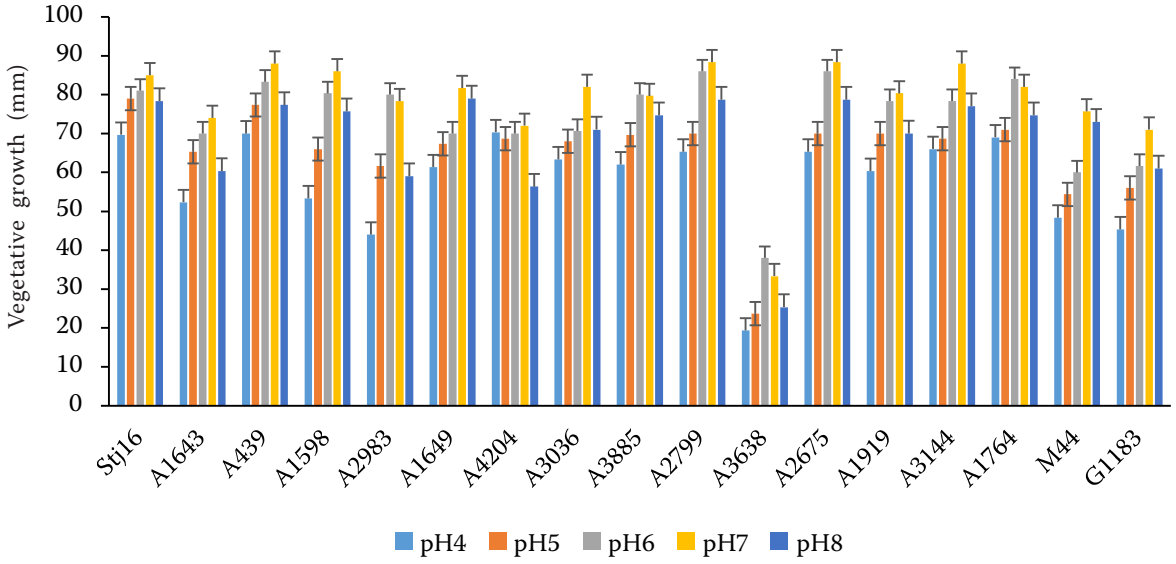

Figure 8. Effect of the different pH levels on the mycelial growth of the T-DNA insertional mutants of *Colletotrichum gloeosporioides*

The error bars represent the SD of the average from three different biological replicates

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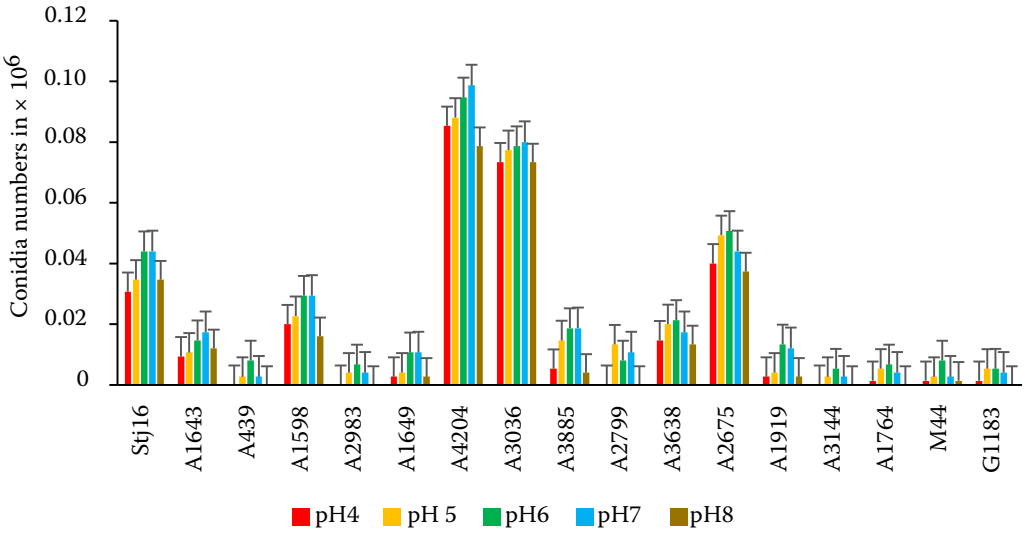

Figure 9. Influence of the pH on the conidia production of seventeen isolates of *Colletotrichum gloeosporioides.*

The error bars represent the SD of the average from three different biological replicates

we evaluated the different medium against the colony growth and sporulation of the insertional mutants of *C. gloeosporioides* and found that the mycelial growth of the described isolates was perfectly optimum mycelial growth on the PDA culture medium, followed by PCA and V-8.-

However, although the PDA medium stimulated good mycelial growth, the conidia production was moderate when compared with the CM, YDA, and RBA culture media. PDA medium has been used as the base culture medium for the isolation, purification and growth of filamentous fungi (Denoyes-Rothan et al. 2003; Schiller et al. 2006; Xie et al. 2010). In the current study, we explored the fact that all the isolates produced limited vegetative growth and the conidia in the oat meal (OM), rose bengal agar (RBA) and onion agar (OA) medium. The water agar (WA) significantly influences the colony growth and conidia production of this fungus.

The temperature fluctuation also disturbs the development of the *C. gloeosporioides* strains. The growth of the tested pathogen gradually increased with an increase in the temperature. The maximum mycelial growth of A2983, A1919,

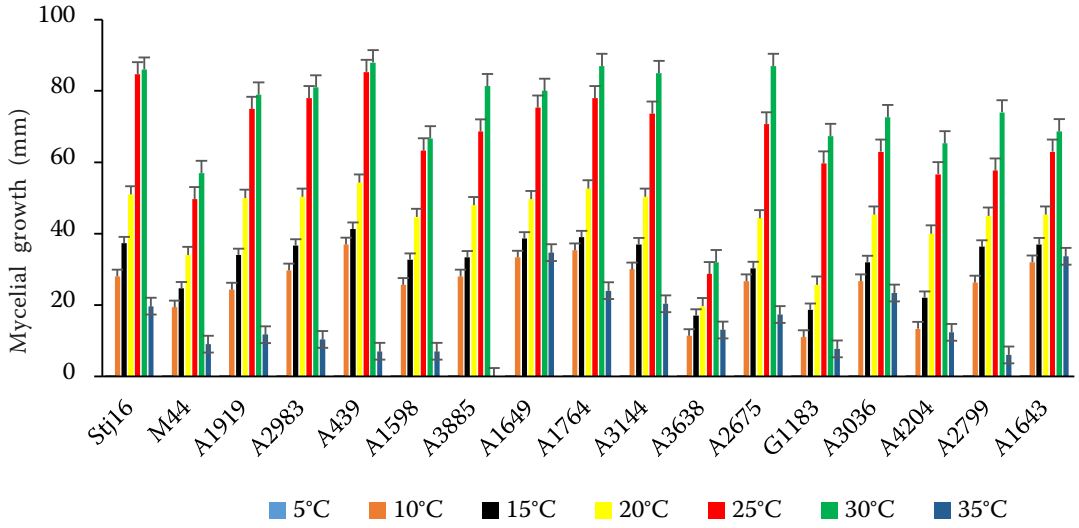

Figure 10. Effect of the different temperatures on the mycelial growth of the *C. gloeosporioides* isolates

The error bars represent the SD of the average from three different biological replicates

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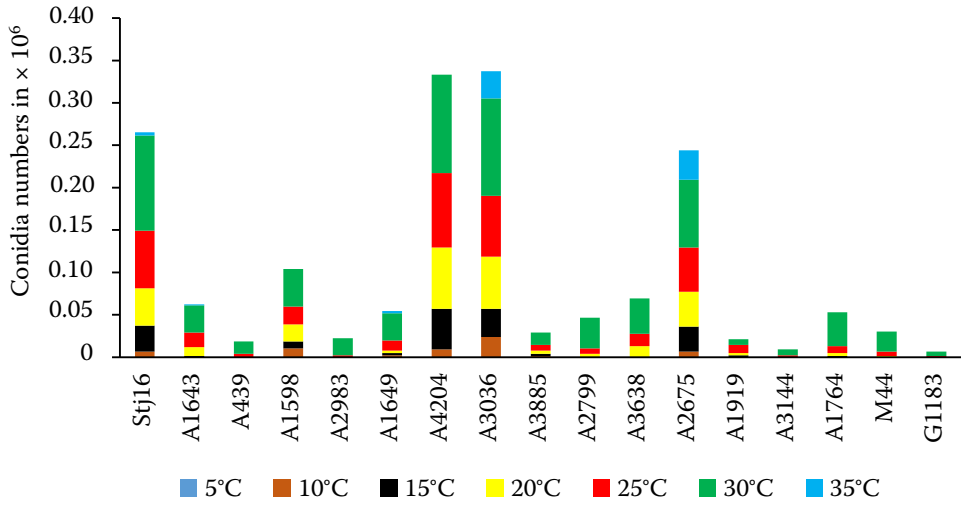

Figure 11. Temperature fluctuation effect on the sporulation of the T-DNA insertional mutants of *Colletotrichum gloeosporioides.* The data are the consequences of three different biological replicates

A3144 and A2799 was reached at 25 °C and 30 °C, average mycelial growth was reached at 15 °C and 20 °C, and gradually declined thereafter at 35 °C. All the insertional mutants found no development at 5 °C. Fernando et al. (2000) described that the anthracnose causing fungi Colletotrichum was found to be more active at 25 °C ± 2 °C. These consistent results found that the different species of Colletotrichum isolated from the mango, banana and passion fruit produce good vegetative growth and sporulation at 28 °C (Talhinhas et al. 2002). None of the insertional mutants grew at 5 °C and only the wild isolate "Stj16", A1649, A1764, A1643 developed at 35 °C. The comparable finding indicated that Colletotrichum isolated from the avocado, papaya and banana also persisted at 36 °C (Peres et al. 2008). The optimum germination of the *C. gloeosporioides* isolates causing the anthracnose disease in coffee occurred at 21 °C–29 °C (Kenny et al. 2012) and its hyphal growth was maximal at 21 °C (Varzea et al. 2002). The pH is also an important factor which plays a significant role in the growth and differentiation of microorganisms. In this research study, the pH variation had no remarkable effect on the mycelial growth and the germination of the seventeen studied mutants: the mycelial growth, sporulation and germination of all the strains performed optimally at pH 6 and 7. In 2014, De Costa and Chandima (2014) reported that C. gloeosporioides, the causal agent of the banana anthracnose, has an optimal growth at pH 4.5.

Light has a profound effect on the vegetative and reproductive development of *C. gloeosporioides.* The present study revealed that the maximum mycelial growth was observed when the inoculated plates were exposed to 12-hour light and dark intervals, followed by continuous light and continuous darkness, continuous light and an alternate 12 h light and 12 h dark cycle, which yielded the maximum sporulation of the isolates of *C. gloeosporioides* when compared to continuous darkness. Latinovic and Vucinic (2002) and Mello et al. (2004) reported relative findings about *C. gloeosporioides* isolated from olive plants. The anthracnose disease caused by numerous species of the genus *Colletotrichum* and particularly *C. gloeosporioides* represents one of the major destructive fungal diseases of apples. Therefore, the obtained phenotypic defective and pathogenic mutants also provide a platform that deciphers the molecular mechanism controlling this disease for cultures of apple plants. In summary the of this study, we identified the suitable culture media and favourable environmental conditions appropriate for the rapid growth and sporulation of the fungus *C. gloeosporioides,* the information generated will facilitate the pathological and molecular research on the fungus and the disease it causes.

CONCLUSION

In the present study, four pathogenicity related mutants (A4204, M44, A3638 and A1598) were screened out through an *in-vitro* pathogenicity test, they lost their pathogenic capability completely,

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and the insertion mutant A1919 slightly produced symptoms on the wounded apple fruits. The subcellular location of A2799 were analysed through the observation of the GFP with fluorescent microscopy, it was found that it is present in the peroxisomes of both the mycelia and conidia cell. The A4204 insertion mutant obviously increased the conidia production compared with the wild isolate "Stj16", which means that the A4204 gene controls the sporulation. Four insertion mutants (M44, A2983, A3144 and G1183) strongly decreased the sporulation of the fungi, which means that they positively control the production of the conidia. The biological study indicates that: (1) the RBA medium decreases the mycelium growth, but it can increase the sporulation for some of the isolates; (2) most of the isolates fit into a large pH range (from pH 4 to pH 8), but mutant A4204 does not grow well at pH 4 and pH 8, and mutant M44 just has optimum growth at pH 8; (3) the 12-h light and 12-h dark conditions stimulate the sporulation for most of the tested mutants, but the A1764 mutant sporulated more in regular dark conditions. Based on the–biological study, we found the best growing and sporulation conditions for all the tested isolates. All the mutants and "Stj16" grew vigorously at 25 °C–30 °C, for "Stj16", it produced the highest number of conidia at 30 °C compared with the other temperatures, it explained that the pathogen can cause disease severity in warm and humid conditions in the field. The insertion mutant A3638 has poor colony growth in all the media, but it keeps its sporulation and pathogenicity, it means this gene positively controls the vegetative growth. The information generated will facilitate further molecular research study on this devastating fungus.

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Received: May 19, 2020
Accepted: January 15, 2021