An alternative inoculation technique of *Colletotrichum gloeosporioides* on mango for early anthracnose tolerance screening[1]

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Una técnica alternativa de inoculación de Colletotrichum gloeosporioides en mango para la detección temprana de la tolerancia a la antracnosis

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ABSTRACT:

The importance of having a technique that allows an efficient expression of symptoms of anthracnose is based on the early differentiation of cultivars and the optimization of genetic, material and financial resources. The objective of this research was to generate an alternative inoculation technique for Colletotrichum gloeosporioides on mango for early anthracnose tolerance screening. On this technique was optimized some of the most relevant components such as isolate virulence, conidial density, the inoculum deposition on leaves and using of surfactants. The study was carried in Iguala, Mexico, during the production cycles 2015-2016. *C. gloeosporioides* was biologically and culturally characterized. Gro and Sin monosporic strains were isolated from leaves, flowers, fruits and branches with anthracnose symptoms from commercial mango orchards located in Guerrero and Sinaloa states, Mexico. These strains show mycelial growth at 2.2 and 2.1 cm of diameter per day, spore density of 4.3x106, 3.9x106 conidia/ ml, germination of 27 and 26%, virulence, with incubation period 4.5 and 4.1 days after inoculation, incidence of 90 and 92% and severity of 3.2 and 3.5 cm of diameter. The highest incidence and severity values with the lowest incubation period, was obtained using Gro isolate (1x105 conidia/ml) and polyoxyethylene-20-sorbitan monolaurate as spreader-sticker inoculated on the abaxial surface on detached young leaves, 15-20 days old, with a soft brush and incubated under dark condition. This inoculation technique allowed the optimal expression of *C. gloeosporioides* virulence in mango leaves and could be incorporated as a tool in the early differentiation of tolerance and susceptibility among cultivars.

Resumen:

La importancia de contar con una técnica que permita una expresión eficiente de síntomas de antracnosis se basa en la diferenciación temprana de cultivares y a la optimización de recursos genéticos, materiales y financieros. El objetivo de esta investigación fue generar una técnica de inoculación alternativa para Colletotrichum gloeosporioides en mango para la detección temprana de la tolerancia a la antracnosis. En esta técnica se optimizaron algunos de los componentes más relevantes como la virulencia de los aislamientos, la densidad de conidios, la deposición de inóculo en hojas y el uso de surfactantes. El estudio se realizó en Iguala, México, durante los ciclos de producción 2015-2016. *C. gloeosporioides* fue caracterizado biológica y culturalmente. Los aislamientos monospóricos Gro y Sin, fueron obtenidos de hojas, flores, frutos y ramas con síntomas típicos de antracnosis procedentes de huertos comerciales de mango ubicados en los estados de Guerrero y Sinaloa, México. Estos aislamientos presentaron un crecimiento micelial de 2,2, y 2,1 cm de diámetro por día, densidad de esporas de $4,3 \times 10^6$, $3,9 \times 10^6$ conidios/ ml, germinación de 27 y 26%, virulencia, con un período de incubación de 4,5 y 4,1 días después de la inoculación, 90 y 92% de incidencia y severidad de 3,2 y 3,5 cm de diámetro. Los valores de incidencia y severidad más altos y los períodos de incubación más bajos se obtuvieron con el aislamiento Gro (1×10^5 conidios/ml), en un medio de monolaurato de polioxietileno-20-sorbitán sobre la superficie abaxial de hojas jóvenes desprendidas, de 15-20 días de desarrollo, con el empleo de una brocha de pelo suave e incubado en condiciones de oscuridad. Esta técnica de inoculación permitió la expresión óptima de virulencia de *C. gloeosporioides* en hojas de mango y podría incorporarse como una herramienta en la diferenciación temprana de la tolerancia y susceptibilidad entre cultivares.

Palabras clave: conidios, esporas, *Mangifera indica* , técnicas de aislamiento.

Introduction

Mexico is the main mango exporter worldwide and placed among the first five producer countries (FAO, 2016). Anthracnose is one of the most important diseases of this crop, mainly caused by *Colletotrichum gloeosporioides* (Penz.) (Ploetz and Prakash, 2000). *C. gloeosporioides* has cosmopolitan distribution in mango producing regions, affects most severely in flowering, fruit setting, and postharvest (Carreon et al., 2010). The initial symptoms in leaves consist of small dark spots with chlorotic halo; lesions can grow and coalesce to reach ± 1.0 cm in diameter. Flowers show small brown lesions on the primary and secondary axis that result in a blight of the panicle (Prusky, 1994). Anthracnose cause losses ranging from 50 to 100% and severity from 70 to 80% in young fruits (8-15 mm), with high environmental humidity and inadequate agronomical management (Arauz, 2000; Prusky et al., 2009). An inoculation technique of *C. gloeosporioides* that permit to determine optimal conditions for infection, development of symptoms and to study the relationship of the pathogen with their hosts may be useful for testing resistance in new varieties of mango and to establish strategies to manage this disease (Denoyes-Rothan et al., 2003; Hernández et al., 2005; Moral and Trapero, 2009). Most of the studies on etiology and epidemiology of *C. gloeosporioides*, report inoculation methods of the pathogen in flowers and fruits; however, research on inoculation techniques of leaves is limited (Acosta et al., 2001; Biggs and Miller, 2001; Gutiérrez-Alonso et al., 2003; Moral and Trapero, 2009) and operates with different efficiency degrees, often being inconsistent. These protocols generally consider several components of a technique frequently applied in separate form as inoculum densities and deposition methods, additives, phenological stages and incubated conditions (Paéz, 1997). The objective of this research was to generate an alternative inoculation technique for *C. gloeosporioides*, on mango for early antracnose tolerance screening.

Materials and methods

Study site

The study was carried out in the Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, in Iguala, Guerrero, Mexico (18° 25'N, 99° 35'W, 731 masl); during the production cicles 2015-2016. The mango plants were 18-month-old, grafted with "Ataulfo" cultivar,

established in plastic pots in a nursery covered with transparent plastic (caliber 600) and a polypropylene mesh, 50% shade. The plants were fertilized by applying in a weekly basis Steiner nutrient solution (1.0 l per plant poured in the soil and 1.0 ml/l sprayed to the canopy) and watered to field capacity every third day. Temperature, relative humidity and photoperiod in the nursery were registered every two hours with a Hobo® data logger, model U12.

Strains obtainment and culture characterization

Leaves, flowers, juvenile fruits and annual vegetative branches with anthracnose symptoms were collected from commercial mango orchards located in the states of Oaxaca, Guerrero, Colima and Sinaloa, Mexico. Plant tissue fragments, approximately 1 cm long with typical symptoms, were disinfected with sodium hypochlorite 1% for 2 min, rinsed three times with sterile water and dried with a sterile absorbing towel before being transferred to Petri plates containing potato-dextrose-agar medium. Plates were incubated at 28 °C for seven days under alternating 12:12 h light-dark conditions.

To evaluate growth rate, sporulation and germination, a 2 cm diameter PDA disc from each strain was transferred to individual plates with PDA and incubated at 22-25 °C during seven days. The diameter of the colony was measured (cm) with a digital vernier. Five replicates were done per isolate (treatment). To estimate sporulation, five 2 cm diameters mycelial, were collected per isolate from seven-day-old cultures and placed in a blender with 50 ml sterile distilled water, and mixed during four 2 s intervals to promote conidia and acervuli detachment. The content filtered through a metallic mesh (200 mesh/in^2) was collected into a glass beaker and adjusted to 100 ml with sterile distilled water. The conidial suspension was vortexed for 20 s and concentration of conidia/ml was estimated in five preparations using a Neubauer Chamber. The spore viability was quantified by depositing one drop (50 µl) of inoculum ($1x10^5$ conidia/ml) in an excavated slide with cover slide and placed in a Petri plate on a wet sterile paper disc to avoid dehydration. Slides were incubated at 24 ± 3 °C under 12:12 light and dark conditions. Was considered as some germinated conidia when these emitted a germinative tube longer than his body, and were quantified after 48 h through light microscopy. Five slides (experimental units) were quantified per isolate (treatment) in each condition.

Additives and conidial viability

Gro isolate at a density of $1x10^4$ conidia/ml was mixed with 0.1% of polyoxyethylene-20-sorbitan monolaurate, 0.1% isoparaffin commercial mixture of ethoxylated polyglycol and 0.1% aryl-polyethoxy ethanol alcohols. Sterile distilled water was included as control. One drop of each suspension was placed in an excavated slide, covered and placed in a Petri plate on a wet sterile paper disc to avoid dehydration, and incubated at 24 ± 3 °C in continuous darkness. Conidial germination was quantified at 24 and 48 h through light microscopy.

Isolates virulence in detached leaves

The incubation period (*Pi*), incidence and severity of each isolate were evaluated using the next detached leaf inoculation: vegetative buds were marked in 18-month-old cv. "Ataulfo" plants in the nursery to observe foliar development; the leaves were cut when they were 20-days-old, then were disinfested with sodium hypochlorite at 0.5% for 30 s, rinsed three times with sterile distilled water and blotted in a laminar flow cabinet in asepsis conditions. Previously, 25 x 35 x 10 cm (L, W, D) plastic containers were disinfested with sodium hypochlorite (5%) and cleaned with alcohol (95%), the containers were left in a laminar flow cabinet until dry. Then, the bottom of the container was covered with sterile towels humidified to saturation with sterile distilled water.

The disinfested and dried mango leaves were placed on the wet towels inside the plastic container. Half of each leaf (respect to the central nerve) was inoculated by depositing (without wounds) three drops (50 ml) of a conidial suspension ($1x10^5$ conidia/ml) on the adaxial and abaxial surface (separately) of the leaves. Each drop was placed separately, equidistant, approximately 35 mm from the central nerve of each leaf to generate individual lesions. The plastic containers with the inoculated leaves were covered with transparent polyethylene and incubated at 24 ± 3 °C, under alternate 12:12 dark: light conditions and 100% relative

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humidity for eight days. Five leaves were placed in each container (experimental units), and five containers per strain (treatments) were evaluated. The *Pi* and incidence were quantified. The severity was determined eight days after inoculation (dai) measuring each lesion diameter (cm) with a digital vernier.

Inoculum densities

Cv. "Ataulfo" 20-days-old mango leaves were inoculated with Gro isolate due to its high virulence, using $1x10^4$, $1x10^5$, $4x10^5$ and $1x10^6$ conidia/ml with polyoxyethylene-20-sorbitan monolaurate (0.1%) using the detached leaf technique previously described. In this test, the inoculum was deposited only on the abaxial surface of the leave. The plastic containers with the inoculated leaves were incubated at 24 ± 3 ℃ under alternate 12:12 dark: light conditions and 100% relative humidity. Five leaves per container (experimental unit) were inoculated and four containers (replicates) by treatment (doses) was evaluated. The *Pi* and incidence were quantified. The severity was evaluated after eight days, considering the diameter of each lesion (cm) with a digital vernier.

Statistical analysis

For each one of the trials a completely randomized design was used and analysis of variance (GLM) and mean tests (LSD, p = 0.05) were performed with SAS v.9.3 (SAS Institute Inc, 2012).

Plant nursery inoculation

Nursery 18-month-old mango plants cv. "Ataulfo" with similar growth and vigor characteristics were selected for this study. Vegetative buds were marked and when the leaves had twenty days of development they were detached and disinfested with 0.5% NaCl solution for 30 s, rinsed three times with autoclaved distilled water using a hand-held sprayer and left to dry for 10 min.

Five inoculation procedures were evaluated: 1) manual spraying, 2) contact with a cotton swab, 3) contact with a soft brush (camel hair), 4) contact with cotton cloth, and 5) direct mycelium contact.

Inoculation was done on both abaxial and adaxial surface of the leaves inoculating on one half of the leaf, considering the central foliar nerve as reference. A $1x10^5$ concentration of conidia/ml suspended in polyoxyethylene-20-sorbitan monolaurate (0.1%) of *Gro* isolate was used. Five leaves per plant (experimental unit) were inoculated and four plants (replicates) per treatment (inoculation procedure) evaluated. Inoculation was done before sunset at 18:00 h ($\pm$ 300-450 lm), inoculated leaves were covered with a dark plastic bag during 12 h and the plants were kept in a nursery covered with shading mesh (70% shade) until symptoms appeared. *Pi* and incidence were determined. Severity was evaluated 15 dai through digital images, estimating the affected area (%) of each leaf using the GIMP 2.0 software for Windows®.

In the nursery, the temperature oscillated between 29 and 31 ℃, the relative humidity 85-90%, and the photoperiod 12 ± 1 h light. These variables were registered every two hours with a Hobo® data logger, model U12.

A completely randomized block statistical design was used, and a variance analysis and mean separation (LSD, p≤ 0.05) were done using the SAS v.9.3 (SAS Institute Inc., 2012).

Re-isolating strains

Vegetative tissues with symptoms of anthracnose collected from the experimental units of both trials (detached and attached leaf technique) were fragmented into pieces of approximately 1 cm in length, isolating was made using a monosporic culture technique and the species was corroborated using taxonomic keys of Ainsworth et al. (1973), Barnett and Hunter (1998) and Bailey and Jeger (1992).

Results

Isolates, virulence and susceptible tissue

Monosporic strains Gro, Col, Oax, Sin and Tux were isolated from infected mango tissue; Gro and Sin showed the higher growth rate, sporulation density and germination (Table 1). These factors correlated with virulence (Estrada-Valencia et al., 1997; Montesinos-Matías et al., 2011). It was observed that Gro and

Sin isolates induced the highest incidence and severity in detached leaves (Table 2) (p<0.05). The highest conidial germination was registered in the dark; incubation under no light favors the inoculum and exerts the highest pressure on mango tissue (Willocquet et al., 1996). 12 h darkness period followed by 12 h natural light stimulated conidial germination and favors the establishment of infection. Inoculation on the abaxial surface without wounds resulted, in a short incubation period and highest incidence and severity (Table 2).

TABLE 1

Mycelial growth and sporulation density of five *C. gloeosporioides* isolates collected from mango orchards, after seven days of incubation on PDA medium under 12:12 light-dark conditions and conidial germination rates at $1x10^5$ conidia/ml density. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Isolate	Diameter of colony (cm)	Conidia/ml in micelial growth disc (2.0 cm Ø)	% Germination in light (300 lm)	% Germination in dark
Gro	2.27 a	4,367,436 a	27.33 a	61.00 a
Sin	2.10 a	3,992,519 a	26.50 a	64.75 a
Tux	2.45 a	572,439 b	14.75 b	38.50 b
Col	1.45 b	1113 c	15.50 b	21.75 c
Oax	1.45 b	4875 c	17.33 b	22.33 c
LSD (5%)	0.32	$1.7x10^6$	4.07	9.53
CV (%)	10.76	63.24	9.95	11.19

Values with the same letter in the same column are not significantly different (LSD p<0.05); CV= variation coefficient; LSD= least significance difference / Valores con la misma letra en la misma columna no son significativamente diferentes (LSD p<0,05); CV= coeficiente de variación; LSD= diferencia menor significativa.

Cuadro 1. Crecimiento micelial y densidad de esporulación de cinco aislamientos de *C. gloeosporioides* colectados en huertos de mango, después de siete días de incubación en medio PDA bajo condiciones de luz-oscuridad 12:12 y tasas de germinación conidial a una densidad de $1x10^5$ conidios/ml. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

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TABLE 2

Virulence of five isolates of *C. gloeosporioides* inoculated at a density of 1×10^5 conidia/ml on the adaxial and abaxial mango leaves surfaces cv. "Ataulfo" twenty-days-old, using the detached leaf inoculation technique. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Isolate	Incubation period (dai)[1]		% of Incidence[1]		% of severity[1]	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
Gro	5.12 DC	4.57 DE	81.25 B	90.50 A	2.57 B	3.27 A
Sin	5.25 DC	4.17 E	77.50 BC	92.25 A	3.12 AB	3.50 A
Col	7.25 A	6.50 AB	71.25 CD	71.25 CD	0.87 C	1.02 C
Oax	7.25 A	6.00 BC	58.75 F	65.50 ED	0.75 C	1.30 C
Tux	7.30 A	6.33 AB	60.00 EF	63.33 ED	0.77 C	0.76 C
Control[2]	0	0	0	0	0	0
LSD (5%)	0.98		6.35		0.6	
CV (%)	11.34		5.68		18.87	

[1] Phytosanitary parameters eight days after inoculation (dai); [2] Control inoculated with sterile distilled water; values with the same letter in the same column are not significantly different (LSD p<0.05); CV= variation coefficient; LSD= Least significance difference / Parámetros fitosanitarios ocho días después de la inoculación (dai); [2] Control inoculado con agua destilada; valores con la misma letra en la misma columna no son significativamente diferentes (LSD p<0,05); CV= coeficiente de variación; LSD= diferencia menor significativa.

Cuadro 2. Virulencia de cinco aislamientos de *C. gloeosporioides* inoculados a una densidad de 1×10^5 conidios/ml sobre las superficies adaxial y abaxial de hojas de mango cv. "Ataulfo", de veinte días de edad, con la técnica de inoculación de hojas desprendidas. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

Additives, conidial viability and inoculum densities

The highest germination rate was observed when conidia was suspended in water and polyoxyethylene-20-sorbitan monolaurate (Table 3). Due to its tensoactive, adherence and dispersal properties, polyoxyethylene-20-sorbitan monolaurate has been used by researches to maximize the time and uniformity of inoculum contact with the foliar tissue; being a non-ionic tensoactive material, it allows cohesion rupture of the sprayed drops (Frias et al., 1995). Three inoculum densities (1×10^5, 4×10^5 y 1×10^6) ($p\leq0.05$) resulted in the highest severity of anthracnose with the short incubation period on detached mango leaves (Table 4). In this study was used 1×10^5 conidia/ml density, since some of the isolates had lower sporulation rate and this concentration was more feasible to obtain in the laboratory from a smaller number of fungal colonies.

TABLE 3

Germinative viability of *Gro* isolate (*Colletotrichum gloeosporioides*) *in vitro* at a density of $1x10^5$ conidia/ml using three additives with different tensoactive, adherence and dispersal properties after incubation for 24 and 48 h. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Additives	% Germination at 24 h[1]	% Germination at 48 h[1]
Conidia + Water	33.10 A[2]	74.75 A[2]
Conidia + polyoxyethylene-20-sorbitan monolaurate (Tween 20®) (0.1 %)	33.35 A	73.00 A
Conidia + aryl-polyethoxy ethanol alcohols (Inex-A®) (0.1%)	20.00 B	42.00 B
Conidia + ethoxylated polyglycol (Soltrol®) (0.1%)	13.27 C	25.25 C
LSD (5%)	3.00	9.79
CV (%)	5.77	8.56

[1] Average of five preparations per treatment, temperature 24±3 °C in darkness; values with the same letter in the same column are not significantly different (LSD p<0.05); CV= variation coefficient; LSD= least significance difference / Promedio de cinco preparaciones por tratamiento, temperatura 24±3 °C en la oscuridad; valores con la misma letra en la misma columna no son significativamente diferentes (LSD p<0,05); CV= coeficiente de variación; LSD= diferencia menor significativa.

Cuadro 3. Viabilidad germinativa del aislamiento de Gro (*Colletotrichum gloeosporioides*) *in vitro* a una densidad de $1x10^5$ conidios/ml con el empleo de tres aditivos con diferentes propiedades tensoactivas, de adherencia y dispersión, después de incubación durante 24 y 48 h. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

TABLE 4

Anthracnose assessment induced by the *Gro* isolate (*Colletotrichum gloeosporioides*) at four conidial concentrations suspended in polisorbato 20 (0.1%) inoculated on the abaxial surface of mango leaves cv. "Ataulfo" twenty-days-old, using the detached leaf inoculation technique. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Conidial concentration (conidia/ml)	Incubation period (dai)[1]	Incidence[1]	% Severity[1]
1×10^4	5.60 A	95.00 A	2.10 A
1×10^5	4.22 B	92.50 A	3.32 B
4×10^5	4.17 B	93.25 A	3.32 B
1×10^6	4.15 B	92.25 A	3.45 B
Control[2]	0	0	0
LSD (5%)	0.73	7.25	0.64
CV (%)	7.58	6.70	10.45

[1] Phytosanitary parameters eight days after inoculation (dai), average of five sheets per treatment, incubation at 24±3 °C and 100% relative humidity, 12:12 light: dark; [2] Control inoculated with sterile distilled water in polisorbato 20 (0.1%); values with the same letter in the same column are not significantly different (LSD p<0.05); CV= variation coefficient; LSD= least significance difference / Parámetros fitosanitarios ocho días después de la inoculación (dai), promedio de cinco láminas por tratamiento, incubación a 24±3 °C y 100% humedad relativa, 12:12 luz: oscuridad; [2] Control inoculado con agua destilada en polisorbato 20 (0,1%); valores con la misma letra en la misma columna no son significativamente diferentes (LSD p<0,05); CV= coeficiente de variación; LSD= diferencia menor significativa.

Cuadro 4. Evaluación de antracnosis inducida por el aislamiento *Gro* (*Colletotrichum gloeosporioides*) en cuatro concentraciones de conidios, suspendido en polisorbato 20 (0,1%) e inoculado en la superficie abaxial de hojas de mango cv. "Ataulfo", de veinte días de edad, con la técnica de inoculación de hojas desprendidas. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

Inoculation methods and severity evaluation

The symptoms development were different on each technique, on detached leaf technique appeared circular spots of fast growth, on the other hand, in the attached leaf technique could be observed leaf anthracnose typical symptoms, with small angular spots that can coalesce and limited by nerves (Figure 1). The detached mango leaf inoculation technique (Tables 2 and 4) was effective to evaluate the pathogenicity (Inc) and virulence factors (*Pi*, severity) of the *Gro* experimental Isolate, which showed the highest parasitic aptitude to be included in the inoculation technique generated in this study. It was observed that 20-day-old leaves were the most susceptible to anthracnose (compared against leaves >25-days-old, data not shown). The use of a soft brush, followed by cotton swabbing, and conidial spraying, were the most successful way to inoculate and reproduce adequately anthracnose symptoms on the abaxial surface of the plant´s leaves under nursery conditions (p<0.05) (Table 5). The GIMP 2.0 software for Windows® allowed to determine the real proportion of the damaged tissue and avoid over or underestimation that frequently occurs when a pictorial diagram is used. A similar method of digital image analysis was used satisfactorily by Sauceda-Acosta et al. (2015) to evaluate the severity of *Puccinia triticina* in wheat through Image J 1.0 software for Windows®.
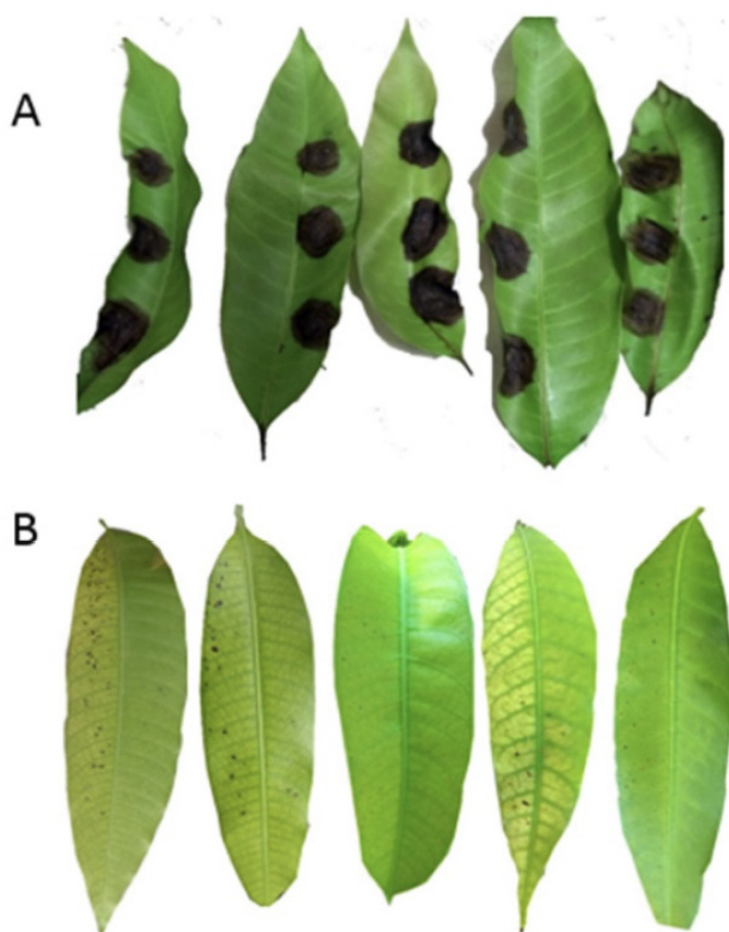
FIGURE 1

Anthracnose (*C. gloeosporioides*) symptoms on mango leaves. A. Detached leaf technique and B. Attached leaf technique. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Figura 1. Síntomas de antracnosis (*C. gloeosporioides*) en hojas de mango. A. Técnica de hoja desprendida y B. Técnica de hoja adherida. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

TABLE 5

Anthracnose assessment induced by five inoculum deposition methods of *Colletotrichum gloeosporioides Gro* isolate suspended in polisorbato 20 (0.1%) at density 1x10^5 conidia/ml on 20 days old mango leaves cv. "Ataulfo" in nursery conditions. Unidad Académica de Ciencias Agropecuarias y Ambientales of the Universidad Autónoma de Guerrero, Iguala, Mexico, 2015-2016.

Method of deposition	Incubation period (dai)[1]	Incidence[1]	% Severity[1]
Contact with a soft brush (camel hair)	6.57 B	49.75 A	5.30 A
Contact with a cotton swab	6.25 B	29.50 B	2.62 B
Manual spraying	8.30 B	19.00 C	2.16 BC
Contact with cotton cloth	12.45 A	8.05 D	1.37 C
Direct mycelium contact	12.40 A	5.42 D	1.45 C
Control[2]	0	0	0
LSD (5%)	3.27	6.83	0.98
CV (%)	18.21	15.45	19.64

[1] Phytosanitary parameters fifteen days after inoculation (dai), average of five leaves per treatment, incubation between 29 and 31 ºC, relative humidity between 85 and 90% and photoperiod 12±1 h light; [2] Control inoculated with sterile distilled water in polisorbato 20 (0.1%); values with the same letter in the same column are not significantly different (LSD p<0.05); CV= Variation coefficient; LSD= Least significance difference / Parámetros fitosanitarios quince días después de la inoculación (dai), promedio de cinco hojas por tratamiento, incubación entre 29 y 31 °C, humedad relativa entre 85 y 90%, y fotoperiodo de 12±1 h luz; [2] Control inoculado con agua destilada en polisorbato 20 (0,1%); valores con la misma letra en la misma columna no son significativamente diferentes (LSD p<0,05); CV= coeficiente de variación; LSD= diferencia menor significativa.

Cuadro 5. Evaluación de antracnosis inducida por cinco métodos de deposición de inóculo de *Colletotrichum gloeosporioides* aislamiento *Gro* suspendido en polisorbato 20 (0,1%) a una densidad de 1x10^5 conidios/ml, en hojas de mango cv. "Ataulfo" de 20 días de edad en condiciones de vivero. Unidad Académica de Ciencias Agropecuarias y Ambientales de la Universidad Autónoma de Guerrero, Iguala, México, 2015-2016.

Discussion

The isolates *Gro* and *Sin*, obtained from states of Guerrero and Sinaloa, Mexico, showed better virulence factors and virulence (Tables 1 and 2), similar results were reported by Gutiérrez-Alonso et al. (2001), who characterized *C. gloeosporioides* strains from several mango producing regions in Mexico through morphology, morphometry, germination rate, growth, sporulation and pathogenicity studies. Nelson et al. (2014) and Moral et al. (2009) found that incubation in the darkness at 25 °C favored successful infection and the presence of symptoms in mango fruits inoculated with *Colletotrichum* spp. In this study 12 h darkness stimulated conidial germination and establishment of the infection. Than et al. (2008), Moral et al. (2009), and Nelson et al. (2014) reported that using a 12 h photoperiod resulted in successful infections and typical anthracnose symptoms in several hosts.

Polyoxyethylene-20-sorbitan monolaurate in conidial suspensions of *C. gloeosporioides* to inoculate mango fruits resulted in the highest germination rate (Gutiérrez-Alonso et al., 2001). Even though, conidial spraying with water induced high germination values, it will be better to use a surfactant to avoid rapid dehydration

and prolong the viability of the inoculum, as well as to improve the quality infection. The results showed that using a conidial density of $1x10^5$ conidia/ml induced adequately disease symptoms. This result was similar to the one reported by Alemu et al. (2014), who obtained typical symptoms when they inoculated mango fruits with the same density of *C. gloeosporioides* conidia to evaluate the antifungal activity of botanical extracts, and partly coincided with Kuc and Richmond (1977), who found no significant differences in anthracnose (*C. gloeosporioides*) incidence and severity when they inoculated mango fruits with densities of $1x10^5$ and $1x10^7$ conidia/ml.

The detached leaves inoculation method was useful to evaluate the virulence of the experimental *Colletotrichum gloeosporioides* isolates. The abaxial surface of the leaves was more susceptible, probably because cuticle in adaxial side are harder and contain thicker layers of wax that contribute to loss of inoculum by spill, this was already documented on bean crop attacked by *C. lindemuthianum* (Tu, 1986). The leaves of 15-20 days of age were highly susceptible as documented by Rojas-Martinez et al. (2008), who induced symptoms of anthracnose by inoculating mango leaves of fifteen days of development. Did not found published literature or references about susceptibility of phenological stages of mango leaves to anthracnose; however, it has been documented differentiated susceptibility of the host organs (leaves, fruits) to pathogens infection according to its age (Espinosa et al., 2004). Younger leaves may show most severe symptoms of powdery mildew (*Oidum mangiferae*) on mango (Sinha et al., 2002) and promote more germinated conidia and larger sporulated lesions of *Uncinula necator* in *Vitis vinifera* (Doster and Schnathorst, 1992).

In addition, it is convenient to emphasize the great differences found between the inoculation experiments, where to inoculating in the same type of tissue but with and without being adhered to the plant completely contrasting symptoms were found. The foregoing can be explained by the influence of the plant's defense systems and the conditions in which each trial was developed; the above has been previously documented, Stintzi et al. (1993), for example, described the proteins related to plant pathogenesis (PR´s) and their role in the defense against pathogens.

This optimized protocol is particularly useful in research projects where it is necessary to assure optimal expression of parasitism and virulence of the pathogen, as well as, reproduce the anthracnose with highest efficiency and quality in order to more precisely measure the disease development and the beneficial effect of control treatments. This is also true in genetic mango improvement programs that consider early evaluation of varieties tolerance to *C. gloeosporioides* in the vegetative stage (varieties screening) when it is not possible to evaluate the incidence and severity parameters in reproductive tissues due to the plant phenology. It is relevant to consider that hosts that are highly susceptible to anthracnose in the vegetative stage will have higher epidemic rates due to subsequent reinfections and will show greater severity in the reproductive stages.

Conclusion

Placing the inoculum ($1x10^5$ conidia/ml) suspended in polyoxyethylene-20-sorbitan monolaurate (0.1%) with soft brush on the abaxial surface of leaves under dark conditions, was the best integrated option to consistently reproduce symptoms of anthracnose with the highest efficiency in leaves of mango plants in nursery conditions. This inoculation technique allowed the optimal expression of the virulence of *C. gloeosporioides* in mango leaves and could be incorporated as a tool in the early differentiation of tolerance and susceptibility among cultivars.

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Notes

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