EFFECT OF SYSTEMIC RESISTANCE INDUCTORS ON PR´S ENZYMATIC ACTIVITIES AND SEVERITY OF MANGO ANTHRACNOSE ‡

[EFECTO DE INDUCTORES DE RESISTENCIA SISTÉMICA EN LA ACTIVIDAD ENZIMÁTICA DE PR´S Y SEVERIDAD DE ANTRACNOSIS DEL MANGO]

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SUMMARY

Background: anthracnose (*Colletotrichum gloeosporioides*) is an important disease in mango (*Mangifera indica*) due to its worldwide distribution and inducting of severe epidemics in vegetative and reproductive states, causing significant losses. In nature, plants produce defense mechanisms to protect themselves from biotic and abiotic factors by increasing the synthesis of secondary metabolites and enzymes involving processes such as the accumulation of phenolic compounds, β-1, 3-glucanases and chitinases. **Objective:** in the present work, the increase in the accumulation of phenolic compounds and enzymes related to pathogenesis β-1, 3-glucanases and chitinases was evaluated as a function of inducers of systemic resistance acquired: acibenzolar-S-methyl, salicylic acid and sodium phosphonate (phosphite). **Methodology:** the applications of treatments were initiated when the plants were approximately 2 years old (6 months of acclimation), the treatments were applied via foliage, the foliage sampling for the enzymatic analysis was performed taken at 0, 5, 10, 20, and 30 days after inoculations (dai) and the variables evaluated were: phenolic compounds concentration, enzymatic activity of β-1,3-glucanasess (GLU) and chitinases (CHI) by chromatographic methods, also the incubation period, incidence, severity were evaluated. **Results:** analysis of variance, growth dynamics and correlation showed that those treatments where a resistance inducer was applied had significantly lower incidence and severity levels than the control, as well longer incubation periods. The dynamics of phenolic compound concentration and enzymatic activity were similar in all treatments except for control, observing peaks at 5 and 20 days after application. In addition, a significant correlation between the variables: area under the progress curve of the accumulation of phenolic compounds and activity of β-1, 3-glucanases and chitinases with the anthracnose incidence, severity and incubation period could be observed. **Implications:** in future studies it is recommended to analyze the behavior of treatments in multiple cultivars. **Conclusion:** these results contribute to the mango agronomic management in the region as precedent in the search to low environmental impact alternatives for diseases management, also contributing to the determination of disease's etiology.

Keywords: *Mangifera indica; Colletotrichum* spp.; inoculation; incidence; SAR.

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RESUMEN

Antecedentes: la antracnosis (*Colletotrichum gloeosporioides*) es una enfermedad importante en el mango (*Mangifera indica*) debido a su distribución mundial y por inducir epidemias severas en estados vegetativos y reproductivos, causando pérdidas significativas. En la naturaleza, las plantas producen mecanismos de defensa para protegerse de los factores bióticos y abióticos al aumentar la síntesis de metabolitos secundarios y enzimas que involucran procesos como la acumulación de compuestos fenólicos, β-1, 3-glucanasas y quitinasas. **Objetivo:** en el presente trabajo, se evaluó el aumento de acumulación de compuestos fenólicos y enzimas relacionadas con la patogénesis β-1, 3-glucanasas y quitinasas en función de aplicaciones de inductores de resistencia sistémica: acibenzolar-S-metilo, ácido salicílico y fosfonato de sodio (fosfito). **Metodología:** las aplicaciones de los tratamientos se iniciaron cuando las plantas tenían aproximadamente 2 años (6 meses de aclimatación), los tratamientos se aplicaron a través del follaje, el muestreo del follaje para el análisis enzimático se realizó a los 0, 5, 10, 20 y 30 días después de las inoculaciones (dai) y las variables evaluadas fueron: concentración de compuestos fenólicos, actividad enzimática de β-1,3-glucanasess (GLU) y quitinasas (CHI) por métodos cromatográficos, también se evaluaron el período de incubación, incidencia y severidad. **Resultados:** el análisis de varianza, dinámicas de crecimiento y correlación mostraron que aquellos tratamientos en los que se aplicó un inductor de resistencia tenían niveles significativamente más bajos de incidencia y severidad que el control, así como períodos de incubación más largos. La dinámica de concentración de compuestos fenólicos y la actividad enzimática fueron similares en todos los tratamientos, excepto en el control, observando picos a los 5 y 20 días después de la aplicación. Además, se pudo observar una correlación significativa entre las variables: área bajo la curva de progreso de la acumulación de compuestos fenólicos, actividad de β-1, 3-glucanasas y quitinasas con la incidencia de antracnosis, severidad y período de incubación. **Implicaciones:** en futuros estudios se recomienda analizar el comportamiento de los tratamientos en múltiples cultivares. **Conclusión:** estos resultados contribuyen al manejo agronómico del mango en la región como precedente en la búsqueda de alternativas de bajo impacto ambiental para el manejo de enfermedades, contribuyendo también a la determinación de la etiología de la enfermedad.

Palabras clave: *Mangifera indica*; *Colletotrichum* spp.*;* inoculación; incidencia; RSA.

INTRODUCTION

Mango (*Mangifera indica* L.) is native from India and is the most economically important species of the family Anacardiaceae (Galán, 2009). It is cultivated in most tropical and subtropical areas of the world and represents the third tropical fruit of economic importance (Galán, 2009). Between 1990 and 2010, world production increased by more than 50% (FAOSTAT, 2018; Galán, 2009). India is the first producer, while Mexico represents the first exporter (FAOSTAT, 2018). The level of production in tropical agriculture depends on factors such as germplasm quality, environment, incidence of pests and diseases, and others of economic, social and technical nature (Norse *et al.*, 1992; Oerke *et al*., 2006). Anthracnose (*Colletotrichum gloeosporioides* Penz.) Is one of the most important pre and post-harvest crop diseases and its present in all producing areas around the world (Arauz, 2000; Galán, 2009). The disease is more severe with high relative humidity and abundant rainfall. It infects leaves, flowers, fruits and branches of all ages, can cause losses of 50 to 100% in fruits produced with high environmental humidity and poor agronomic management (Arauz, 2000; Monteon *et al.*, 2012; Mora *et al.*, 2002). It is important to consider that some hosts that are highly susceptible to anthracnose in the vegetative stage, will have higher epidemic rates because in polycyclic pathogens, the number of secondary infection cycles usually determines the intensity of epidemics in the field which triggers greater severity in critical stages (Trivoli *et al.*, 2006). In nature, plants usually remain healthy, due in part to the manifestation of various defense mechanisms (Browning, 1980). The plant resistance to pathogens often depends on whether it can recognize it at the beginning of the infection and counteract the attack with physical barriers or through biochemical reactions that produce toxic substances to the pathogen or creating conditions that inhibit its development (Andersen *et al.*, 2018). Resistance of this type (induced) occurs when one or more of these substances reach a concentration sufficient to inhibit the progression of infection (Cornide *et al.*, 1994; Díaz-Puentes, 2009). The increase of this resistance includes the increase in the synthesis of secondary metabolites and enzymes, which lead to the development of resistance around infection sites, signals that are transferred to nearby sites and then to the entire plant (Passardi *et al.*, 2005). The defensive response involves processes such as accumulation of phenols, reactive oxygen species and enzymes such as phenylalanine ammonia-lyase (Cornide *et al.*, 1994). Also, the increase in the expression rate of resistance genes that encode proteins related to pathogenesis (PR's) such as β-1,3-glucanases and chitinase, high levels of production of low molecular weight secondary

metabolites with antimicrobial activity as phytoalexins (Agrios, 2005; Passardi *et al.*, 2005; Vivanco *et al.*, 2005). Systemic acquired resistance (SAR) is an active, systemic, broad-spectrum defense response that is associated with high PR´s gene expression (Hammerschmidt and Smith-Becker, 1999). In most cases, SAR is equally effective against fungi, bacteria, viruses or nematodes, regardless of the inducing organism (Díaz-Puentes, 2009, 2012). Research indicates that salicylic acid (SA) is the molecule that has shown the highest evidence of involvement in SAR pathways (Mauch-Mani and Metraux, 1998). Thus, SAR induction generally correlates with increases in SA accumulation both locally and systemically (Díaz-Puentes, 2012). There are not many studies that demonstrate the efficacy of this type of products in the management of tropical fruit diseases, particularly in mango, so the objective of the present study is to determine the effects of applications of Acibenzolar S-Methyl (BTH), salicylic acid (SA) and potassium phosphite in the incubation period, incidence and severity of anthracnose, as well as in the enzymatic activity of chitinase (CHI) and β-1, 3-glucanase (GLU) and phenolic compounds (FEN) in mango plants inoculated with *C. gloeosporioides*.

MATERIALS AND METHODS

Plant material

The research was carried out in the Academic Unit of Agricultural and Environmental Sciences of the Autonomous University of Guerrero, located in the Iguala Gro. Valley, Mexico (18° 25' N, 99° 35' W, at 731 masl) during spring 2016 to summer 2017. Two years old mango plants cv. Ataulfo were used and established in plastic pots and maintained in a nursery with polyethylene mesh (50% shade); plants were fertilized weekly with the Steiner nutrient solution to the soil (1 L per plant) and to the foliage (aspersion) and irrigated every two days until field capacity, pesticide applications were not carried out to avoid interference problems. In the nursery, the temperature oscillated between 29 and 31°C, the relative humidity 85-90% and the photoperiod 12 ± 1 h light. These variables were registered every two hours with a data logger HOBO® Model U12.

Pathogen

Colletotrichum gloeosporioides was isolated from leaves, flowers and branches of mango with typical symptoms of anthracnose and cultured on potato dextrose agar (PDA) at 25 °C. The spores were obtained from a PDA mycelium growth disc from *Gro* strain of 15 days of age suspended on sterile distilled water containing 0.05% (v/v) Tween-80 in polyoxyethylene-20-sorbitan monolaurate (0.1%), were shaker in a blender and prepared at $1 \cdot 10^5$ spores/mL with the aid of a Neubauer chamber.

DNA extraction of the fungus and identification of species

DNA extraction was done from the mycelium of monosporic (monoconidial) of *Gro* strain grown and taxonomically identified; DNA extraction was performed by the protocol described by Mahuku (2004) modified for this study. For the identification of the species of *Colletotrichum* sp. associated to mango anthracnose, were used a polymerase chain reaction (PCR) technique using specific primers of ITS (Internal Transcribed Spacers) ITS1-5.8S-ITS2 ribosomal DNA (rDNA). Were used the primers CgInt (5'GGCCTCCCGCCTCCGGGCGG3 ') for *C. gloeosporioides* and CaInt2 (5'GGGGAAGCCTCTCGCGG3') for *C. acutatum*, each combined with the universal primer ITS4 (5'TCCTCCGCTTATTGATATGC3 ') of the conserved region of gene 25/28S rDNA (Afanador-Kafuri *et al.*, 2003; Salazar *et al.*, 2012; Sreenivasaprasad *et al.*, 1996). A mixture of 2.5 µL of buffer *Taq* 10X, 0.16 mM dNTPs, 0.4 mg * mL^{-1} BSA, 1.5 U Taq Polymerase, 1.7 mM $MgCl_2$, 1.6 µM of each primer, approximately 30 ng of step DNA and sterile HPLC nanopure quality water reaching a total volume of 25 µl was used for each PCR. The amplification conditions consisted of an initial denaturation at 94 °C for 5 min; 30 denaturation cycles at 94 °C for 45 s (120 s for initial), alignment at 57 °C for 60 s (240 s for initial); initial extension at 72 °C for 120 s (240 s for the initial); and a final extension of 72 °C for 7 min in a Hybaid thermocycler (Hybaid Ltd., Teddington, England), in all cases positive controls were included for *C. gloeosporioides* and *C. acutatum*, as well as a negative control without DNA. For visualization and analysis of the amplified product were prepared agarose gels with 1.5% ethidium bromide 0.5 µg/mL and 0.5X TBE buffer (10mM Tris, 20mM Borate, 1mM EDTA) with GelRed (2 µl/50mL), electrophoresis conditions were 100 volts and 35 milliamperes for 90 minutes and visualization of the amplified PCR product after the electrophoresis were on a Kodak EDAS 290 photodocumentation system.

Analysis of RAPD profiles

Once the banding patterns were obtained from the samples, all the unequivocally identifiable bands generated with each initiator were counted and double state of presence / absence to create a binary matrix data matrices were prepared. Subsequently, a similarity matrix was generated from the binary data using the DICE similarity coefficient in the SIMQUAL program of the NTSYS-PC package (Rohlf, 2000). Cluster analysis was performed by constructing cluster matrices with the UPGMA (Unweighted Pair Group Arithmetic Mean) method in the SAHN program of the NTSYS-PC package and a dendrogram based on genetic distances was constructed.

Inoculation

Vegetative buds were marked and when the leaves had 15 days of development were disinfected with 0.5% NaCl solution for 30 seconds, rinsed three times with autoclaved distilled water using a hand-held backpack sprayer (Swissmex®) and left to dry for 10 min. Inoculation was done on both abaxial and adaxial surface of the marked leaves, spreading softly the inoculum ($1 \cdot 10^5$ spores/mL^{-1}) suspended in polyoxyethylene-20-sorbitan monolaurate (0.1%) of *Gro* isolate with a soft brush (camel hair), inoculating only on one half of the leaf, considering the central leaf rib as reference. Inoculation was done before sunset, approximately at 18:00 h (± 300-450 lux), inoculated plants were covered with a dark plastic bag during 12 h and kept in a nursery covered with shading mesh (50% shade) until symptoms appeared. Incubation periods and incidence was determined. Severity was evaluated at 20 days after inoculations (*dai*) through digital images, estimating the affected area (%) of each leaf using the GIMP 2.0 software for Windows®.

Treatments application

The applications of the treatments were initiated when the plants were approximately 2 years old (6 months of acclimation), the treatments were applied via foliage to drip point, varying the volume applied according to the state of development of the plant; in the experiment six treatments that were applied: T1: application of the inducer Acibenzolar-S-Methyl (ASM) at a dose of 100 $\mu g \cdot mL^{-1}$, followed by inoculation of *C. gloeosporioides* 4 days after application (dda); T2: Application of salicylic acid (AS) at a dose of 0.2 mg mL^{-1}, followed by inoculation of *C. gloeosporioides* 4 days dda; T3: Application of potassium phosphonate (phosphite) at a dose of 20 $mL \cdot L^{-1}$, followed by inoculation of *C. gloeosporioides* 4 dda; T4: Application of the fungicide azoxystrobin at a dose of 2 $mL \cdot L^{-1}$, followed by the inoculation of *C. gloeosporioides* 4 dda; T5: Application of a control (Sterile distilled water), followed by the inoculation of *C. gloeosporioides* 4 dda; T6: Application of absolute control (sterile distilled water), without inoculation; In all cases a second application of inducer, fungicide or control treatment was performed 15 days after the first one.

Samples taking

Foliage sampling (20 g) for the enzymatic analysis was performed. Samples were taken at 0, 5, 10, 20, and 30 days after inoculations (*dai*) per experimental unit. The variables evaluated were: phenolic compounds concentration, enzymatic activity of β-1,3-glucanasess (GLU) and chitinases (CHI). The laboratory measurements were made from acetone powder, which was obtained by macerating 20 g of leaves in 250 mL of acetone at 4 °C in a domestic blender, vacuum filtered to promote the separation of acetone and photosynthetic pigments that could interfere with the study.

Phenolic compounds assay

To 0.03 mL of the acetone, the supernatant was added 16.97 mL of deionized water with 1 mL of Folin-Ciocalteu and 2 mL of 20% sodium carbonate. It was shaken and allowed to stand for 2 hours in the dark to read absorbance at 760 nm on a Spectronic 21D spectrophotometer (Milton Roy, USA). Quantification was performed by a standard tannic acid curve and the concentration of total phenolic compounds was recorded as $mg \cdot g^{-1}$ of fresh weight (Waterman and Mole, 1994).

Enzymatic extraction

The enzyme was extracted from 0.1 g of acetone powder dissolved in a buffer solution (50 mM Tris-HCl pH 7.2), in a 1:1 (w/v) ratio, which contained sucrose (10%). Polyvinylpyrrolidone (5%) and β-mercaptoethanol (0.1%). It was homogenized for 50 seconds and centrifuged for 20 min at 17000 rpm at 4 °C. Protein concentration was determined by the Bradford method (Bradford, 1976) on a Spectronic 21D spectrophotometer (Milton Roy, USA), performing the absorbance readings at 595 nm, for which a standard curve of bovine albumin was made from a stock solution of 1 $mg \cdot mL^{-1}$.

Chitinase activity assay

The chitinase activity was determined by the colorimetric method, described by Boller, *et al.* (1983), where colloidal chitin obtained from chitin (Fluka reagent quality) was used as the substrate and the absorbance recorded at 585 nm. To perform the standard curve was used an n-acetyl glucosamine stock solution (1 $mg \cdot mL^{-1}$), from which the dilutions were prepared to which the same procedure was performed. Subsequently, the activity was calculated according to the expression:

$$\text{Enzymatic activity: } (D.O. / t.\ incub) \cdot Cot \ (Vtest/ Venz) \cdot dil$$

Where t. incub is the incubation time of the assay, Vtest and Venz are the test and enzyme volumes, respectively and Cot, the cotangent of the standard curve. The enzymatic activity was expressed as μmoles of product formed / min / mL enzyme (UAE).

β-1, 3-glucanase activity assay

For the determination of this activity, 50 μL of laminarin (β-1,3-glucan) (2 $mg \cdot mL^{-1}$) and 50 μL of the enzyme extract were mixed and incubated for 30 min at 37 °C. Subsequently, reducing sugars formed at 660 nm were quantified according to the method proposed by Somogyi (1952). The standard curve was performed using glucose (1 $mg \cdot mL^{-1}$) as standard, from which solutions with different concentrations were prepared. The calculation and expression of this activity were the same as for chitinase. Specific activity of each of the enzymes was determined according to the expression: specific enzyme activity / protein concentration ($\mu g \cdot mL^{-1}$).

Experimental design and correlation analysis

Trees with similar growth, development and vigor characteristics were used. Five leaves per plant (inoculated) and five plants per treatment (experimental unit) in five blocks (replicates) were used in a completely randomized block statistical design. In order to compare the effect of the factors on the disease, Area under the progress curve (AUPC), growth rate (b^{-1}) and final concentration (y^f) of incubation period, incidence, severity, phenolic compounds (FEN), β-1,3-glucanases activity (GLU) and chitinase activity were calculated for each treatment, by integrating the Trapezoidal method (Campbell and Madden, 1990; Campbell and Benson, 1994).

A normality analysis, variances analysis and means comparison (Tukey, $p \leq 0.05$) were done using the SAS v.9.1.3 (SAS Institute Inc, 2008) statistical software. In order to detect parallel behaviors or the influence of one metabolite on another, or their interactions, correlation tests were run between the different variables included in this study.

RESULTS AND DISCUSSIONS

Molecular characterization

The amplification of ribosomal DNA with specific primers from the DNA obtained from the *Gro* isolates of *Colletotrichum* sp., produced fragments of approximately 450 bp in seven replicates with the combination of primers *Cg*Int (specific for *C. gloeosporioides*) and ITS 4 (Figure 1). On the other hand, the PCR reaction with the first *Ca*Int2 (specific for *C. acutatum*) and ITS4 did not produce amplified fragments. The size of the products corresponds to that reported in the literature, 450 bp for *C. gloeosporioides*. This results, indicated that the isolation *Gro* of *Colletotrichum* spp., obtained from mango in the state of Guerrero, Mexico, corresponds to *C. gloeosporioides*, corresponding to the expected size for amplification with these DNA primers belonging to *C. gloeosporioides* (Alvarez *et al.*, 2004, Salazar *et al.*, 2012; Sreenivasaprasad *et al.*, 1996).

However, a small amount of mycelium coming from of isolates *Gro* was scraped from a seven-day-old culture and the internal spacer region (ITS) of the extracted fungal DNA was amplified with the combination of primers *Cg*Int (specific for *C. gloeosporioides*) and ITS 4. The resulting sequence of 476 and 458 pb was submitted to the NCBI GenBank (Accession MK59194 isolate *Gro* and MK591945 isolate *Gro2*). A blast search of ITS sequences in NCBI revealed that this fungus was *Colletotrichum gloeosporioides* and *Colletotrichum asianum* respectively by 99.7 and 99.5% query coverage.

The similarity dendrogram (Figure 2), based on the Dice coefficient, for Gro isolates of *Colletotrichum* spp, generated from the PCR-RFLP, RAMS technique, amplifying the DNA and amplification with the specific primers CgInt and CaInt2 in combination with ITS 4 primer allowed the confirmation of *C. gloeosporioides* and *C. asianum* as the causative species of anthracnose symptoms in mango foliage as previously reported by Lima, *et al.* (2013), who in the north of Brazil and with the help of a moltilocus analysis of sequences found four species of *Colletotrichum* spp. causing mango anthracnose and similar to that found by Grupta, *et al.* (2010) who identified 25 different isolates

of *Colletotrichum gloeosporioides* that cause mango anthracnose.

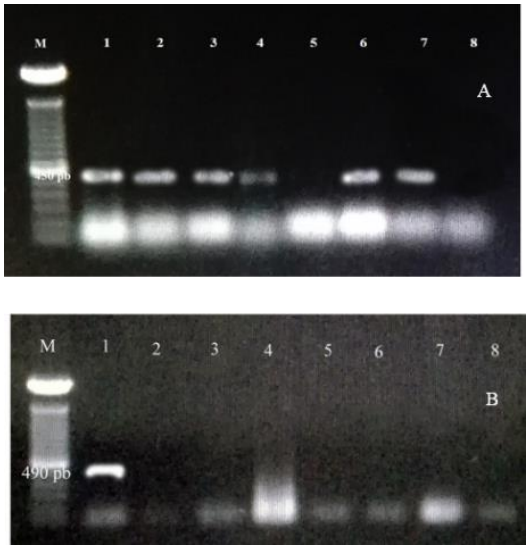

Figure 1. A. Electrophoretic patterns of amplification products of specific ribosomal DNA fragments of the *Colletotrichum* sp. of *Gro* strain using the primers: CgInt - ITS4, M = 100pb marker, lane 1 = positive control of *C. gloeosporioides*, Lane 2-7 = isolates of *Colletotrichum* sp., Lane 8 = negative control; **B.** Electrophoretic patterns of amplification products of specific ribosomal DNA fragments of *Colletotrichum* sp. of *Gro* strain using the primers: *Ca*Int - ITS4, M = 100pb marker, lane 1 = positive control of *C. acutatum*, lanes 2-7 = isolates of *Colletotrichum* sp., Lane 8 = negative control.

Disease development

Systemic resistance induction treatments had a significant effect on the variables: incubation period, incidence and severity (Figure 3). Although in the three variables evaluated, the systemic fungicidal agent obtained the highest control levels, treatment 2 (ASM) presented highly significant differences with the inoculated control without the application of chemical agents, registering a 6-day incubation period and a reduction in the incidence and severity of 40 and 75% respectively (Figure 3). In the particular case of resistance inductors, the ASM stands out over the others. The above was similar to that reported by Lin *et al.* (2011), who informed that applications of Acibenzolar-S-Methyl (BTH) in mango fruits (*M. indica*) cv. Keitt reduced the incidence and diameter of the spots (severity) in both inoculated fruits and natural infections. It was also similar to that found by Cao and Jiang (2006) who, when compared to control, observed a significant decrease in disease incidence and lesion diameter in mature pears inoculated with *Penicillium expansum* or *Alternaria alternata* and treated trees three times with ASM. Other studies have demonstrated this behavior in crops such as tobacco, cucumber, sunflower, banana, and papaya (Friedrich *et al.*, 1996; Mogollón-Ortiz and Castaño-Zapata, 2011; Prats *et al.*, 2002; Zhu *et al.*, 2003). Our results indicate that ASM and SA treatments suppress significantly the anthracnose caused by *C. gloeosporioides* in mango leaves.

Coincident with this research, some reports indicate that Acibenzolar-S-methyl (ASM) acts as an inducer of resistance in many crops to various pathogens, inducing high expression of genes encoding enzymes: peroxidase (POX), phenylalanine ammonium-lyase (PAL), related to pathogenesis proteins PR1-1, PR1-1a, PR-2 (β-1,3-glucanases), PR-3 (Chitinases) and PR-5 that play an important role in the defense against pathogens containing b- 3-glucan and chitin (Cools and Ishii, 2002; Heil and Bostock, 2002; Luna *et al.*, 2012; Mandal *et al.*, 2009a, 2009b; Maxson-Stein *et al.*, 2002; Narusaka *et al.*, 1999).

In relation to azoxystrobin, multiple toxicity reports have been found in earthworms, fish, aquatic invertebrates, aquatic plants, and in addition to having a 12-day field activities re-entry period as a preventive measure of poisoning risk (Cao *et al.*, 2016; Gustafsson *et al.*, 2010; Han *et al.*, 2014; Olsvik *et al.*, 2010; Toumi *et al.*, 2019); in the other hand, ASM, potassium phosphonate and salicylic acid could be more ecological alternative options as they do not have such toxic characteristics. Like in the present work, the potassium phosphonate has been used as an alternative to phytosanitary management in some crops such as banana, papaya, citrus, potato and cauliflower, decreasing levels of incidence and severity in previously inoculated susceptible plants (Bécot *et al.*, 2000; Machinandiarena *et al.*, 2012; Mogollón-Ortiz and Castaño, 2011; Orober *et al.*, 2002; Vawdrey *et al.*, 2007). Similar to our results, multiple researches report that the applications of salicylic acid (SA) and acetylsalicylic acid (ASA) have been shown to be effective in significantly reducing incidence and severity in crops such as strawberry, rose, bean, tomato, etc., increasing the activity of enzymes such as β-1, 3-glucanase, phenylalanine ammonia-lyase and chitinases in pre and post-harvest (Bokshi *et al.*, 2003; Senaratna *et al.*, 2000; Torres-Velásquez *et al.*, 2013; Yao and Tian, 2005).

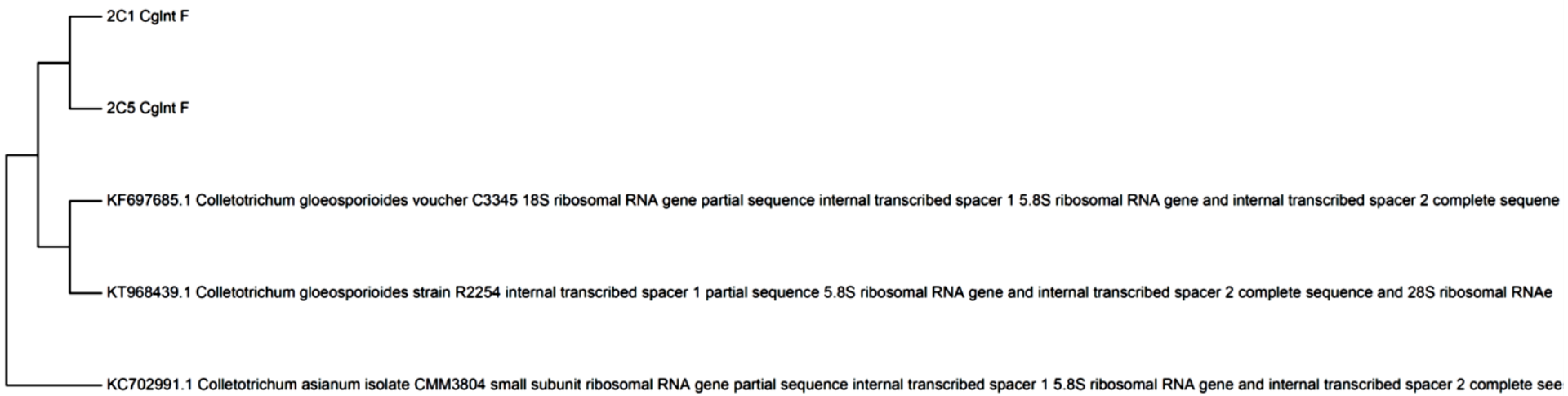

Figure 2. Based on RAPD allele pattern, dendrogram was derived from cluster analysis (UPGMA) showing the relationship among the isolates.

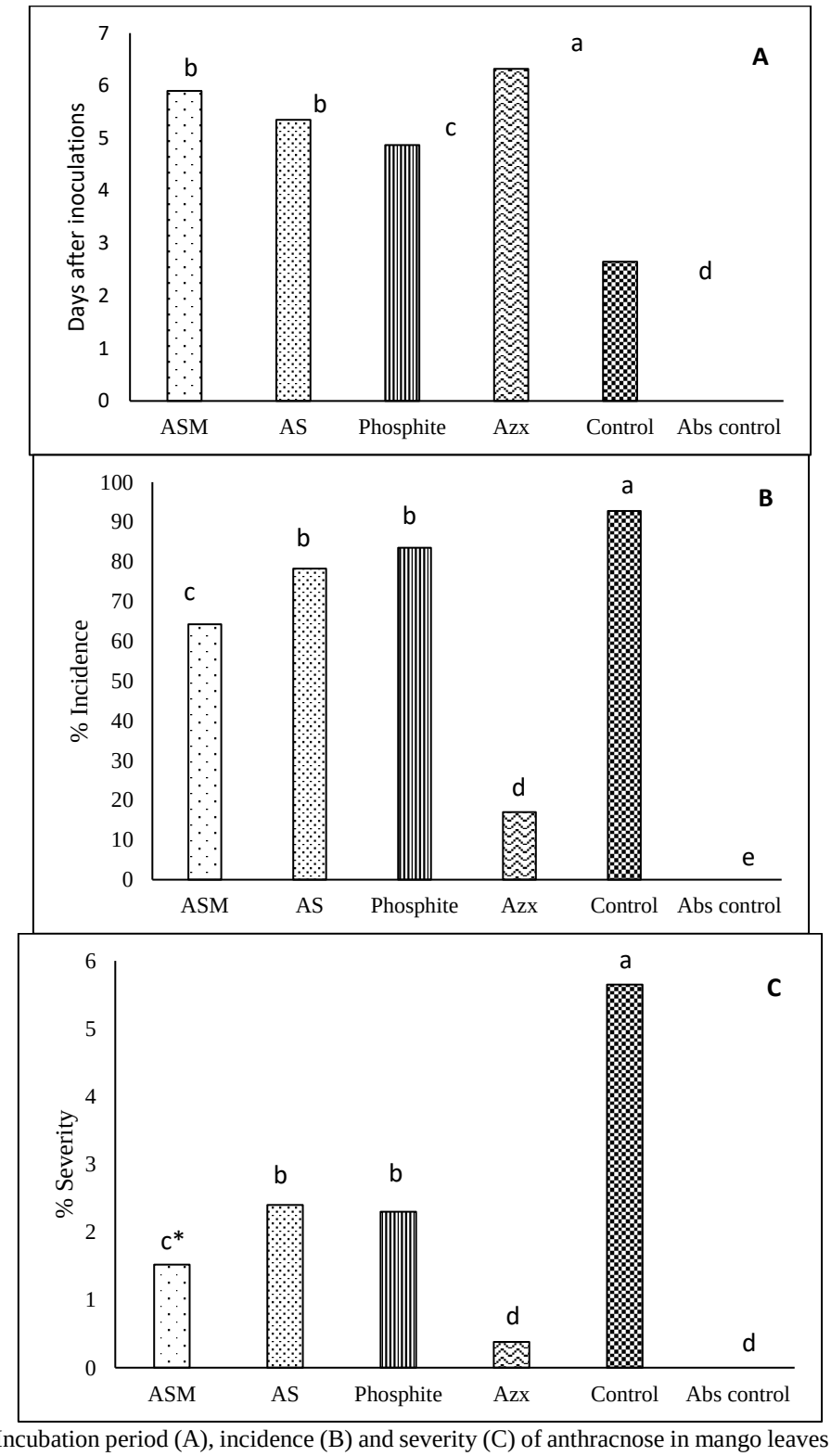

Figure 3. Incubation period (A), incidence (B) and severity (C) of anthracnose in mango leaves (*Mangifera indica*) cv. Ataulfo inoculated with *Colletotrichum gloeosporioides* and under chemical induction systemic resistance treatments; *equal letters in the columns are not significantly different, Tukey's test ($p \geq 0.05$). ASM = Acibenzolar -S- Methyl (100 $\mu g \cdot mL^{-1}$); AS= salicylic acid (0.2 mg mL^{-1}); Phosphite= potassium phosphonate (20 $mL \cdot L^{-1}$); Azx= Azoxystrobin (2 $mL \cdot L^{-1}$); Control= Inoculated control; Abs. control= Absolute control.

Phenolic compounds concentration

It could be observed that the area under the activity progress curve (AUPC), the rate of increase (b^{-1}) and the activity of the final evaluation (y^f) of phenolic compounds concentration in leaves inoculated with *C. gloeosporioides* under systemic resistance induction treatments, was higher when ASM was applied, followed by SA and phosphite, obtaining levels significantly higher than controls, and even that fungicide treatment (Table 1). Results similar to those reported by Lin *et al.* (2011), who applied Acibenzolar-S-Methyl (BTH) treatments on mango cv. Keitt and Zill and found a significant increase of phenolic compounds concentration at 6 and 12 days after application compared to control. Our results also coincided with that found by Prats *et al.* (2002), who reported accumulation of phenolic compounds in ASM treated sunflower leaves, this effect was evident in the third pair of leaves regardless of the time elapsed between treatment and inoculation. The accumulation of these compounds in cell walls and their anti-fungal effect by inhibiting the activity of hydrolytic enzymes has been previously reported (Godman *et al.*, 1986; Nicholson and Hammerschmidt, 1992).

β-1, 3-glucanase activity

This activity was monitored and showed that ASM was the chemical agent that induced the highest activity of the enzyme; however, all inducing treatments were superior to the absolute control (Table 1). Our results were similar to that reported by Cao and Jiang (2006) who, after applying ASM treatments and inoculating pear fruits with *Penicillium expansum* or *Alternaria alternata* showed significantly higher β-1, 3-glucanase activity than the control treatment. Also, our results were coincident with that reported by Zhu *et al.* (2003) who reported an increase in the activity of β-1,3-glucanasess in papaya (*Carica papaya*) plants treated with Acibenzolar-S-Methyl (BTH) and Inoculated with *Phytophthora palmivora*; they also observed that even performing artificial inoculations without systemic resistance inducing applications, the activity of the enzyme increased at considerable levels, which may indicate a systemic response of natural defense, this similar to our results (Table 1).

Chitinases activity

Our results showed significant differences in the variables, area under the activity progress curve (AUPC), rate of increase (b^{-1}) and activity of the final evaluation (y^f), ASM was identified as the best treatment inducing enzymatic activity, Followed by SA and potassium phosphite (Table 1). This was in agreement with what was found by Baysal *et al.* (2003), who in tomato plants (*Lycopersicon esculentum*) induced systemic resistance against *Clavibacter michiganensis* ssp. michiganensis using ASM applications and increasing significantly chitinase activity from 5 to 7 days after application; This has been reported in other crops as papaya (Zhu *et al.*, 2003), sunflower (Serrano *et al.*, 2007), sugar beet (Burketová *et al.*, 1999) and apple (Brisset *et al.*, 2000). Chitinases may act alone or in conjunction with β-1, 3-glucanases as inhibitors of fungal activity, probably because of the ability to hydrolyze the structural components of cell walls, which, in addition, release small fragments of oligosaccharides that can activate defense responses such as the induction of PR proteins and phytoalexins (Ryan and Farmer, 1991).

Dynamics

The dynamics of phenolic compound concentration and β-1, 3-glucanases and chitinases enzymatic activity were obtained. A similar behavior was found in the inducing treatments, ASM induced the highest activity followed by SA and potassium phosphite. In the case of the content of phenolic compounds, a sudden increase was observed five days after the application and then increasing at a lower rate; however, the activity of β-1, 3-glucanases and chitinases were triggered at 20 days probably as an effect of the second application (Figure 4). These results were similar to those reported by Baysal *et al.* (2003) who observed an increase in chitinase activity from the second day after application of ASM in tomato plants (*Lycopersicon esculentum*) inoculated with *Clavibacter michiganensis* ssp. michiganensis, and also to those reported by Zhu *et al.* (2003), who with a single ASM application on papaya (*Carica papaya*) against *P. palmivora* found an increase in the activity of β-1, 3-glucanase for up to 10 days, and similar to that reported by Ishida, *et al.* (2008), they documented an increase in β-1, 3-glucanase activity with peaks at 7 and 14 days after application of acibenzolar-S-methyl in cotton plants inoculated with *Xanthomonas axonopodis* pv. malvacearum. Our results are also coincident with those obtained by Lin *et al.* (2011) who observed peaks of increase in the phenolic compound concentration at 6 and 12 days after an application of ASM as a treatment against mango anthracnose.

Table 1. Area under the activity progress curve (AUPC), growth rate (b^{-1}) and final concentration (y^f) of phenolic compounds (FEN), β-1,3-glucanases activity (GLU) and chitinase activity in mango leaves (*Mangifera indica* L.) cv. Ataulfo inoculated with *Colletotrichum gloeosporioides* and under chemical induction systemic resistance treatments at five evaluation dates.

Phenolic compounds (FEN)			
Treatment	AUPC	b^{-1}	y^f
ASM	84.72 a*	0.17 a*	26.83 a*
AS	62.8 c	0.12 b	22.08 b
Phosphite	72.79 b	0.1 b	20.32 c
Azx	59.03 c	0.08 c	16.29 d
Control	55.21 d	0.08 c	15.57 d
Abs. control	40.73 f	0.03 d	11.3 e
β-1, 3-glucanase activity (GLU)			
Treatment	AUPC	b^{-1}	y^f
ASM	3.24 a*	0.13 a*	1.30 a*
AS	3.10 b	0.11 b	1.11 b
Phosphite	3.06 b	0.08 c	0.96 c
Azx	2.77 c	0.05 d	0.75 d
Control	2.40 d	0.052 d	0.70 d
Abs. control	2.15 e	-0.001 e	0.51 e
Quitinase activity (CHI)			
Treatment	AUPC	b^{-1}	y^f
ASM	0.29 a*	0.65 a*	0.17 a*
AS	0.26 b	0.44 b	0.14 b
Phosphite	0.24 b	0.36 b	0.13 b
Azx	0.20 c	0.18 c	0.08 c
Control	0.16 d	0.17 c	0.05 d
Abs. control	0.09 e	0.06 c	0.02 e

*Averages followed by equal letters in the columns are not significantly different, Tukey's test ($p \geq 0.05$). ASM = Acibenzolar -S- Methyl (100 $\mu g \cdot mL^{-1}$); AS= salicylic acid (0.2 mg mL^{-1}); Phosphite= potassium phosphonate (20 $mL \cdot L^{-1}$); Azx= Azoxystrobin (2 $mL \cdot L^{-1}$); Control= Inoculated control; Abs. control= Absolute control.

Correlation analysis

In the linear regression analysis and Pearson's correlation coefficient determination, could be observed that there is a close relationship between disease development, phenolic compound levels and enzymatic activity. The area under the progress curve of the variables: incubation period, incidence and severity were influenced by synthesis of phenolic compound concentration, β-1, 3-glucanase and chitinases activity (Table 2). These results are similar to that reported by Quecine *et al.* (2008) who found a positive correlation (r= 0.65 - 0.79) between the chitinolytic production of *Streptomyces* spp. coming from citrus and antagonism against *C. sublineolum*, *R. solani*, *G. citricarpa* and *F. oxysporum*. Our results coincide with Metraux and Boller (1986), who in cucumber plants (*Cucumis sativus* L. cv. Wisconsin) found that the increase in chitinase activity was correlated with increased resistance against *Colletotrichum lagenarium* infection. The findings of this study were also coincident with those reported by Shresta, *et al.* (2008), who found a negative correlation between chitinase activity levels and the severity and number of lesions in rice cultivars inoculated with *Rhizoctonia solani*; also Giannakis *et al.* (1998), reported a correlation between resistance and β-1, 3-glucanase activity in vid leaves (*Vitis* spp.) attacked by powdery mildew (*Uncinula necator*); Also, Patil and Widholm (1997), reported a linear correlation (r= 0.74) between endochitinase activity and vigor of tobacco plants (*Nicotiana tabacum*).

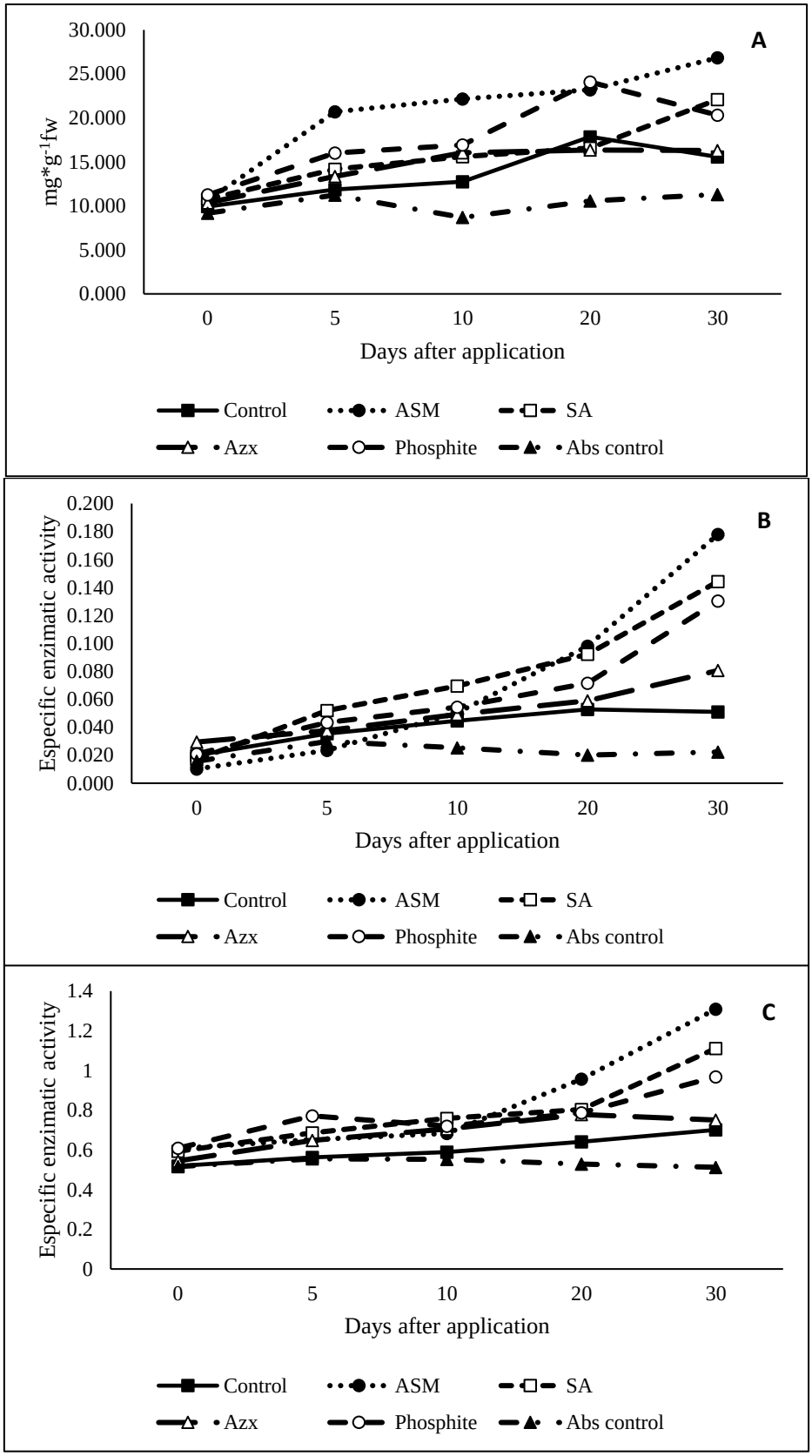

Figure 4. Dynamics of phenolic compound concentrations (A), β-1, 3-glucanase activity (B) and chitinase activity (C) in mango leaves (*Mangifera indica*) cv. Ataulfo inoculated with *Colletotrichum gloeosporioides* and under chemical induction systemic resistance treatments at five evaluation dates. ASM= Acibenzolar -S- Methyl (100 μg·mL^{-1}); AS= salicylic acid (0.2 mg mL^{-1}); Phosphite= potassium phosphonate (20 mL·L^{-1}); Azx= Azoxystrobin (2 mL·L^{-1}); Control= Inoculated control; Abs. control= Absolute control.

Table 2. Pearson's correlation coefficient (r) between the variables: area under the progress curve of incubation period, incidence and severity over phenolic compounds, chitinase activity and β-1, 3-glucanase activity in mango leaves (*Mangifera indica*) cv. Ataulfo inoculated with *Colletotrichum gloeosporioides* and under chemical induction systemic resistance treatments.

	Phenolic compounds	Quitinase activity	β-1, 3-glucanase activity
P. incubation	0.80	0.85	0.90
Incidence	-0.75	-0.84	-0.68
Severity	-0.82	-0.96	-0.87

CONCLUSIONS

In the present study, the species *C. gloeosporioides* and *C. asianum* were identified as causal agents of mango anthracnose, also a direct relationship between the inductor applications, PR´s enzymatic activity increment and disease severity could be observed; on inductors applications, ASM induced the highest level resistance followed by SA and potassium phosphite; besides this, it was possible to document that two applications, resistance inducers are enough to provided protection to the plant in around of 20 days, so it is a viable alternative to be incorporated into a disease integrated management system. These results contribute to the mango agronomic management in the region as precedent in the search to low environmental impact alternatives for diseases management, also, contributing to the determination of disease´s etiology.

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Compliance with ethical standards. Due to the nature of the study, national or international ethical and bioethical standards do not apply.

Data availability. The data are available on abraham.monteon@gmail.com with Dr. Abraham Monteón Ojeda.

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