Article

New causes of rot in rubber sting panel: case *Fusarium* spp.

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Abstract

Moldy rot on the rubber sting panel decreases the yield and regeneration of its bark. The objective was to identify the causative agent of the disease in San Juan Bautista Tuxtepec, Oaxaca and evaluate its in vitro sensitivity to fungicides. To this end, tissue from the diseased sting panel was collected and made insulations. The isolates were characterized in the nutritive media PDA (papa-dextrose-agar), CLA (carnation leaves-agar), FLA (liquid fermentation-agar), CDA (coffee-dextrose-agar) and ZA (carrot-agar). Growth rates were evaluated and pathogenicity tests were carried out in the laboratory and in the field, as well as in vitro sensitivity to Mancozeb 70%, Benomilo, Carbendazin, Propiconazole, Chlorothalonil, and Carboxamide, in 10 treatments and eight repetitions. The results showed four isolates of the genus *Fusarium*, which showed polymorphism according to the culture medium, with radial growth and marked rings. Microconidia and macroconidia dispersed in the mycelium and white, orange and blue sporodochs were observed at 36 days. The growth rate varied with the nutrient medium and the inoculated strain. *F. circinatum, F. lateririum*, *F. decemcellulare* and *F. mangiferae* were identified. In the laboratory pathogenicity tests all inoculated strains were positive and in the field only *F. circinatum* and *F. mangiferae*. The sensitivity bioassay showed fungicide-dependent response and isolation. These results allow us to conclude that the species *F. circinatum*, *F. mangiferae*, *F. lateririum* and *F. decemcellulare* can cause moldy rot in the sting panel of clone IAN 710. This is a first report that involves these species. Its control depends on the species of *Fusarium* and the fungicide used.

Keywords: characterization, culture media, fungicide sensitivity, identification.

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Introduction

The rubber *Hevea brasiliensis* Muell Arg. is a perennial tropical crop well paid, of economic importance at a commercial level for its productive levels and the characteristics of its nature, raw material necessary for the industry, in the manufacture of essential items such as tires, components of engineering and latex products (Picón *et al*., 1997). Although it was established in plantations for more than a hundred years in Mexico and the world (Martínez, 1986), in recent years interest in natural latex plantations has resurfaced since its physicochemical characteristics cannot be obtained from synthetic rubber derived from oil (Frederico *et al*., 1995).

In Mexico there are 33 375 ha (INIFAP, 2014) distributed in the states of Veracruz, Oaxaca, Chiapas and Tabasco, with an estimated production of 62.4 thousand tons and a value of 685.7 million pesos (SIAP-SAGARPA, 2016). The main producing state was Veracruz, with 57.6% of the national production, followed by Oaxaca with 33%. The Tuxtepec region in Oaxaca is the most important with average production areas of 3 ha (Rojo *et al.*, 2011) and constitutes one of the main sources of employment and income for 1 500 producers in that area.

However, despite the unbeatable conditions for commercial rubber production, the country historically maintains a deficit situation and more than 70 thousand tons per year have been imported from Thailand, Indonesia and Malaysia, equivalent to more than 90% of domestic consumption , with a cost greater than 100 million dollars a year (Rojo *et al*., 2011). However, the industrial importance of the plant and its product, in Mexico there is not enough information about the productive potential of the species, the management of the plantations, or the factors that influence latex production (Grist *et al*., 1995; Priyani, 1996).

In young plantations, there are few diseases that cause significant damage, including those caused by *Botryodiplodia theobromae* Pat, *Phytophthora* sp., *Diplodia* sp. or *Colletotrichum* sp. In mature plantations the damages are by *Phytophthora palmivora* Butl. *Corticium salmonicolor* Berk and by *Ceratocystis fimbriata* Elliot (Picón *et al*., 1997). The latter can be dispersed by the wind, the sting blade or by insects such as *Xyleborus hypocryphalus* (Goitia and Rosales, 2001), which decreases the yield and regeneration of its bark.

Therefore, the objective of this study was to corroborate the causative agent of the moldy rot of the sting panel in *Hevea brasilensis* in the common Santa Ursula, Tuxtepec, Oaxaca, as well as perform *in vitro* sensitivity tests with fungicides to inhibit the growth of phytopathogen.

Materials and methods

The present study was carried out in Santa Ursula, municipality of San Juan Bautista Tuxtepec, Oaxaca, which according to the Holdridge system has climatic conditions of warm subtrostingl rainforest. Average annual temperatures of 26 °C. Heavy and abundant rains, annual rainfall of 2 000 to 4 500 mm.

The samples taken were representative of the plantation. For the collection of the material, a deep cut was made in the bark of the tree with the previously sterilized sting tool, until penetrating the xylem infected by the fungus, the samples were processed in the phytopathology laboratory of the Faculty of Agrobiology 'Presidente Juárez' UMSNH.

Preparation of culture media

For isolation and characterization of the isolated culture media used papa-dextrose-agar (PDA), water-agar (AA), coffee-dextrose-agar (CDA), carrot-agar (ZA) and spezieller-nährstoffarmer-agar (SNA) recommended by Leslie and Summerell (2006) for the analysis and characterization of the genus *Fusarium* and by Beever (1969) modified by Christen and Raimbault (1991), which is a medium of liquid fermentation-agar (FLA) for *Ceratocystis fimbriata* (Palma-Sandoval, 1993).

Obtaining insulations

The phytopathological techniques described by Trigiano *et al*. (2004); Agrios (2005), samples were seeded in ½XPDA culture medium and incubated at 28 °C in the dark, hyphae tips. For identification by fixed preparations, morphological was compared with the keys of Barnett and Hunter (1998); Leslie and Summerell (2006). Monosporic cultures were performed and stored in sterile 25% glycerol at -70 °C for use.

Cultural and morphological characterization of *Fusarium* isolates

Cultural characterization was performed in 1XPDA medium. 20 µL of the conidial suspension was inoculated in 25% glycerol. The characteristics of the type and thickness of the mycelium, color of the colony (front and back), agar pigmentation, presence and color of the sporodoch, presence of clamidospores were recorded, as well as the possible presence of hyphae coiled at 15, 30 and 60 days.

The morphological characterization was in CLA medium, 5 µL of the suspension was inoculated, at 10 days the length and width of 50 macroconidia and 50 microconidia of each isolation was measured, the shape, type of foot and apical cell were determined in the macroconidia, as well as the number of septa and presence of sporodochia, the shape, arrangement and type of phyloid was determined from the microconidia, along with the presence and disposition of clamidospores and rolled hyphae if possible.

Growth rate

5 µL of the suspension was inoculated in paperless SNA media, which were incubated for 7 days at 25 °C in the dark. From the colony a 6 mm diameter disc was transferred to the center of a Petri dish with ZA, FLA, CDA and PDA media with sterilized rubber stem pieces, incubated for 72 h and recorded the growth rate (mm), with the data obtained, the analysis of variance was performed with the Statgraphics Centurion XVI program (Statpoint, 2013). Five repetitions were established. The experiment was performed twice.

Pathogenicity tests

According to Dhingra and Sinclair (1985), 60 stems of *Hevea brasiliensis* from clone IAN-710 with 2.5 cm wide and 35 cm long were extracted from the field, disinfested with 5% sodium hypochlorite, the tips were sealed with vinyl paint and for its moved laboratory and processing were wrapped in moistened paper. Under aseptic conditions, they were washed again in 2% sodium hypochlorite. For the inoculation, 10 mm discs were placed with PDA medium with the isolate on stems with and without wound, which were covered with transparent tape. The stems were placed inside moist chambers. Five repetitions were established.

The field pathogenicity test was performed on healthy IAN-710 clone trees of 2.5 and 4.5 years of age, the concentration of conidia varied with the isolated Tux 01 (10.76), Tux 02 (3.0), Tux 03 (1.56) and Tux 04 (8.50) $x10^6$ respectively. 200 mL of each suspension was sprinkled on a wound similar to that of commercial crops, on the soft outer bark. Four repetitions were performed. The treatments were distributed in randomized blocks. The severity assessment was performed four days after inoculation with a visual scale, where: A= bark damaged; B= visible rot; C= visible mycelium; D= necrosis; and E= absence of damage.

Bioassay of *in vitro* sensitivity of phytopathogens to fungicides

Under the Dhingra and Sinclair (1985) technique of moistening filter paper, on Whatman No. 4 paper discs of 6 mm in diameter, the products Benomilo (2 g), Carbendazin (3 mL), Mancozed 70% (4 g), Mancozed 70% (1 kg/6 L regional control), Propiconazole (2.5 mL), Carboxamide (12 g), Chlorothalonil (12 g) and the combinations Chlorothalonil + Carboxamide (12 g), Benomyl + Mancozed 70% (2 + 4 g) recommended by AGI-Hule Papaloapan Region (2012) and distilled water (absolute control).

The discs were impregnated with 50 µL of the solution. Ten treatments were established per isolate including a regional control and an absolute. Each treatment had eight repetitions distributed completely randomly. The experimental unit was a disk. The response variable was the mycelial growth of the isolates, this was recorded after 24 h and until the mycelium of the control treatment touched the filter paper disk. The data obtained from the growth were subjected to an analysis of variance with the Statgraphics Centurion XVI statistical program (Statpoint, 2013).

Results and discussion

Of the isolates made, four isolates were obtained, which by comparison of their morphological characteristics belong to the genus *Fusarium*.

Cultural characteristics of *Fusarium* in 1X PDA culture media with rubber stem pieces, ZA, CDA and FLA

The isolates had a radial growth, with marked rings, 75% of the isolates presented abundant, cottony, loose, aerial mycelium, while the remaining 25% (Tux-01 and Tux-04 in CDA, Tux-02 in PDA and ZA) they had thin mycelium, immersed in the middle. The color of the colony was white, light yellow to brown on the obverse in all isolated and yellow, brown and black on the reverse as

the colony aged, in an outstanding way, the Tux-01 isolate in PDA and CDA and Tux-04 in CDA showed a bluish coloration in the obverse. Pigmentation was observed in the culture medium in 50% of the isolates (Figure 1).

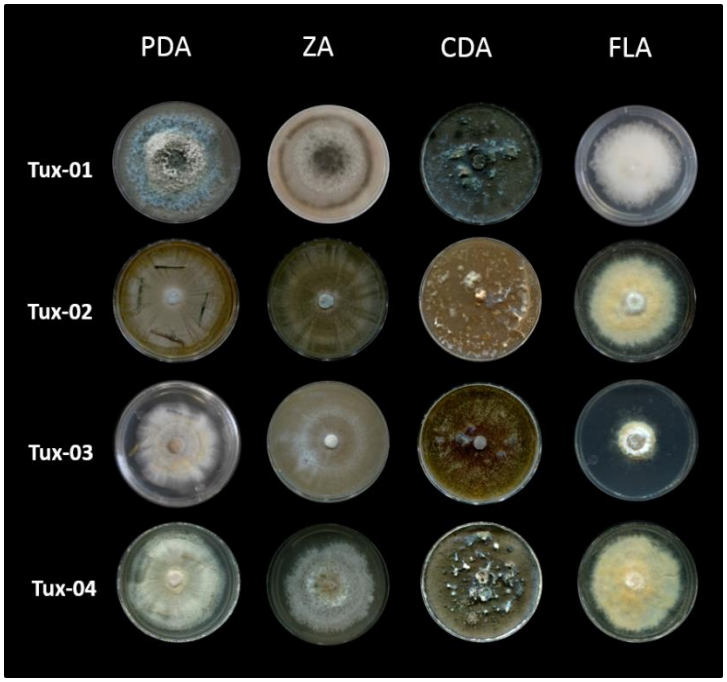

Figure 1. Cultural characterization of *Fusarium* isolates in culture media: PDA with rubber stem pieces, ZA, CDA and FLA.

The temperature, darkness and the culture medium are important factors for the rapid formation of pigments in this genus, which facilitates the fact of observing variability in the intensity of the coloration. In particular, the PDA medium, due to its high carbohydrate content, allows for an ideal development and expression of its cultural characteristics such as shape, color, medium pigmentation and growth range (Leslie and Summerell, 2006).

Morphological characteristics of *Fusarium* in PDA culture medium with rubber stem pieces

All isolates showed the formation of macroconidia, dispersed in the mycelium and in white, orange and blue sporodochs at 36 days. The predominant form was semi-straight with a dorsal curve. The number of septa varied, for Tux-01, Tux-02 and Tux-04 it was three to five, while for Tux-03 it was five to nine. The apical cell observed was papillated and hook-shaped, the basal with a light and clear notch.

The microconidia were present in the four isolates, these were hyaline, usually without septa, some with one or two. Formed in false heads, on mono and polycideids, short and long. Intercalar clamidospores and terminals present in Tux-02, Tux-03 and Tux-04 isolates. Hyphae coiled in Tux-01, Tux-03 and Tux-04.

According to Dhingra and Sinclair (1985) the PDA culture medium can stimulate the formation of clamidospores, which could facilitate their observation. On the other hand, the presence of rolled hyphae can separate *Fusarium* species isolated from different plant materials from pantrostingl regions that belong to the *Gibberella fujikuroi* complex in the *Liseola* section (Leslie, 1995).

Morphological characteristics of *Fusarium* in CLA culture medium

The macroconidia observed were dispersed in the culture medium, abundantly produced in blue and orange sporodochs for Tux-01, orange in Tux-02 and Tux-04 and white in Tux-03 that developed on carnation leaves or medium of culture after 36 days of inoculation (Figure 2A to 2D). The macroconidia were hyaline, semi-direct with a dorsal curve. In the isolated Tux-01 they presented 3 to 4 septa, the apical cell was papillated, the basal was barely notched and measured 39.06 4.4 µm.

In Tux-02 tde 3 to 5 septa, papillary apical cell, the basal with barely notch, 39.4 x 4.4 µm. Tux-03 from 5 to 9 septa, narrow apical cell and basal with clear notch, measured 57.72 x 5.8 µm and Tux-04 from 3 to 4 septa, papillary apical cell, basal with barely notched and 36.4 x 4.9 µm (Figure 2E and F).

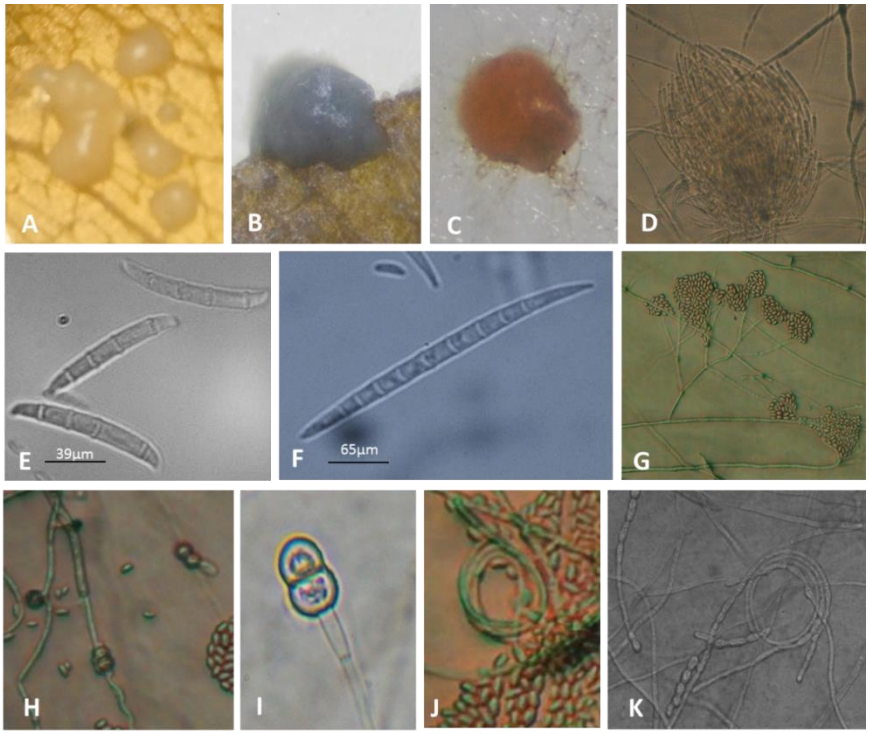

Figure 2. *Fusarium* structures observed in the CLA culture medium. A and B= white and blue sporodochs on carnation leaf; C= orange sporodoquium on the medium; D= sporodoquine seen at 10X; E= macroconidio with four septa; F= macroconidio with nine septa; G= microconidia in false heads; H= intercalar clamidospores; I= terminal clamidospores; J and K= rolled hyphae.

This culture medium is excellent for the formation of *Fusarium* reproductive structures and their preservation, since it decreases the degeneration of the crop due to its low nutrient content (Fisher *et al*., 1982). The macroconidia that occur are usually uniform in size and shape, they occur between 6 and 10 days after inoculating the medium, these peculiarities provide the reason why it is the recommended culture medium for species identification work. The number of septa from 3 to 5 is frequent in species of this genus, however Tux-03 isolation with 5 to 9 (Figure 2E and 2F) coincides with those reported for the species *F. decemcellulare* (Ploetz *et al*., 1996) and *F. lateritium* (Leslie and Summerell, 2006).

The microconidia were formed in false heads arranged in mono or polyphilides (Figure 2G). Ovoid and without septa, the isolated Tux-01 and Tux-02 had one and two septa, while the Tux-03 and Tux-04 also had a cornered shape. The measurements were Tux-01 of 9.48 x 3.08, Tux-02 of 8.2 x 3.2, Tux-03 of 6.52 x 3.2 and Tux-04 of 7.92 x 2.8 µm. Microconidia are not produced by all species of the genus, their presence can be an important character, as well as the size, shape, the conidiogenic cells where they are born or the arrangement they have; the formation of the latter can be affected by the culture medium used.

In *F. decemcellulare* its shape is oval and without septa, usually formed in chains, although they can occasionally be in false heads and on monophalides, in addition to abundant in the aerial mycelium, characteristics that coincide with those observed for the isolated Tux-03 and that they are separated from *F. lateririum* when presented by ellipsoids with zero to three septa, individual, in monophalides and generally absent (Leslie and Summerell, 2006).

Abundant intercalar and terminal clamidospores were observed in Tux-02, Tux-03 and Tux-04 isolates. The presence of these can be a distinctive factor in some species, as well as their disposition, they are usually frequent in crops with SNA and CLA medium more than in other agar surfaces and their formation can take more than six weeks (Leslie and Summerell, 2006), its production can occur in response to changes in temperatures, it is a common survival feature in this genus (Agrios, 2005).

In three of the isolates, hyphae rolled with three and four turns were observed, preferably in aerial mycelium, with the exception of Tux-02 where they were not appreciated (Figure 2). These hyphae are similar to those formed by the species *F. sterilihyphosum, F. circinatum*, *F. pseudocircinatum* and *F. mexicanum* that are the only ones that produce them (Leslie and Summerell, 2006; Jacobs *et al*., 2007; Kvas *et al*., 2009; Otero-Colina *et al*., 2010).

Growth rate for isolates *Fusarium* spp.

The analysis of mycelial growth data at 72 h showed significant statistical differences between them and varied both in the isolates and in the media used. The Tukey averages comparison test $\alpha = 0.05$ for each medium showed that in Tux-01 the best growth was obtained with the ZA medium (13.6 mm) in Tux-02 the ZA media (9.8 mm), CDA (9.4 mm) and PDA (8.8 mm) had a similar growth, for Tux-03 and Tux-04 the best means were ZA (9.9 and 12.6 mm) and CDA (9.9 and 11.7 mm). The lowest growth was observed in the FLA medium in all isolates (Figure 3).

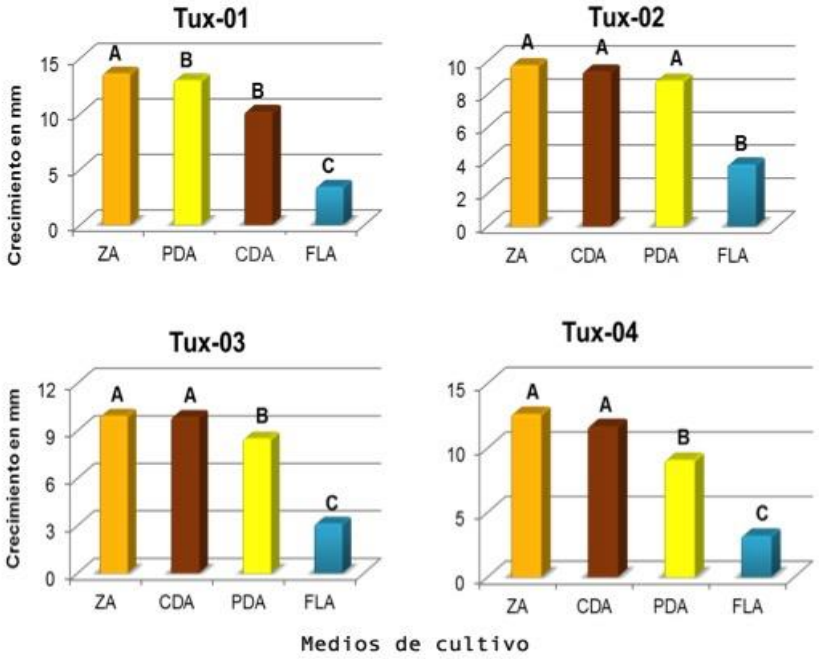

Figure 3. Comparison test of Tukey averages α= 0.05 of *Fusarium* isolates for each culture medium.

The growth rate is a secondary character, the PDA medium is traditionally used and allowed to grow for three days at a temperature of 25 or 30 °C. It usually allows distinguishing fast or slow growing species (Leslie and Summerell *et al*., 2006).

The growth rate for *F. sterilihyphosum* at 25 °C is 4.8 mm day^{-1} (Britz *et al*., 1999), in *F. circinatum* it is relatively fast (4.7 mm day^{-1}) at 20 °C (García, 2011), *F. decemcellulare* is 5-8.3 mm day^{-1} at 25 °C, *F. mangiferae* Britz, Wingfield & Marasas 3.4 mm day^{-1} and *F. lateritium* 2.6-6.6 mm day^{-1} (Leslie and Summmerell, 2006).

The data recorded for the Tux-01 isolates resemble those of the species *F. sterilihyphosum* and *F. circinatum*, with whom it shares the morphological characteristic of rolled hyphae. The other isolates studied are similar to those reported for *F. lateritium*, and *F. mangiferae*, since Tux-02 grew 2.9 mm day^{-1}, Tux-03 (2.8 mm day^{-1}) and Tux-04 (3 mm day^{-1}); however, Tux-03 and Tux-04 have rolled hyphae and the other species do not.

When comparing the cultural, morphological characteristics and growth rate observed in the isolates studied with the descriptions made by (Klitiich y Leslie, 1992; Leslie, 1995; Huss *et al*., 1996, Ploetz *et al*., 1996; Klitiich *et al*., 1997; O'Donnell *et al*., 1998; 2000; Britz *et al*., 1998; 1999; Steemkamp *et al*., 1999a; Leslie and Summerell, 2006; Jacobs *et al*., 2007; Kvas *et al*., 2007; Kvas *et al*., 2009), it could be suggested that the Tux-01 isolate belongs to the species *F. circinatum* Nirenberg & O'Donnell emend. Britz, Coutinho, Wingfield & Marasas, the isolation Tux-02 to *F. lateritium* Nees, Tux-03 to *F. decemcellulare* Brick, and finally Tux-04 to *F. mangiferae* Britz, Winfield & Marasas, although it is evident and indispensable the need to confirm; through the use of molecular tools and multigenic analysis that allow to accurately distinguish each isolation.

Pathogenicity tests

In the test under controlled conditions in the laboratory, it was observed that the inoculated stems without wound did not show any signs of the disease, as did the controls, while the stems with wounds presented the characteristic symptoms within 8 to 10 days. The field pathogenicity test was positive for *F. circinatum* and *F. mangiferae* in all the trees in which they were inoculated. *F. mangiferae* developed a visible rot and mycelium, necrosis coinciding with the expected symptoms at 72 h, while *F. circinatum* did so at 92 h and in 50% of the inoculated trees. The control plants remained free of the disease despite having been injured.

The symptoms described for the disease are similar to those caused by the genus in the trunk of the *Pinus halepensis* pine (García, 2011), Aleppo pine (Garbelotto *et al*., 2007), peach (Jacobs *et al*., 2007), where *F. circinatum* by blocking the vascular bundles of plants, generates a withering of the same, with decrease in size and strength. The damage they cause is easily observed when the bark falls off, under these elongated, dark to black lesions with abundant resin there is presence of mycelium if there is high relative humidity.

The species *F. lateritium* and *F. decemcellulare* did not cause the symptoms of the disease in the field, this contradicts their results in the laboratory, the importance of environmental conditions is transcendent so that the cycle of a disease can be carried out.

Bioassay of in vitro sensitivity to fungicides

The analysis of variance indicated a different response in the presence of fungicides depending on the isolation. The fungicidal isolation interaction showed high statistical significance, as well as the Tukey mean comparison test $\alpha = 0.05$ ($p = 0.0001$), in the effect of the treatments used on the isolates evaluated at 11 days after the bioassay was established (Figure 4).

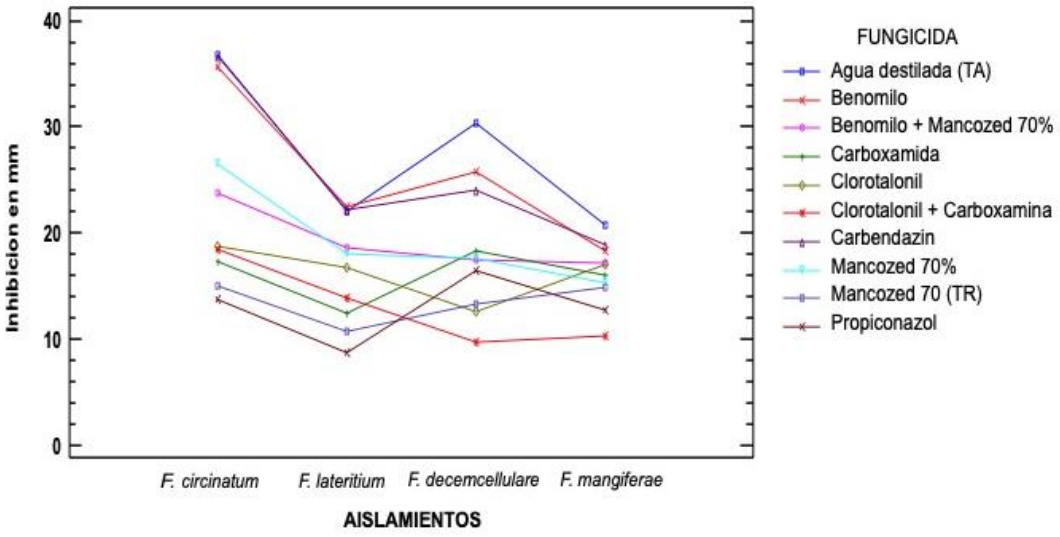

Figure 4. Interactions, action and effect of fungicides, variation on strains of the genus *Fusarium*.

The results of the Tukey α= 0.05 tests for *F. circinatum* showed that the best products for mycelial growth inhibition were Propiconazole, Mancozed (TR), Carboxamide, Chlorotlonyl + Carboxamide and Chlorothalonil. The products with the lowest inhibition were Benomyl, Carbendazin and distilled water. For the *F. lateritium* isolate, the best effects were presented with Propiconazole, Mancozed (TR) and Carboxamide, while Mancozed, Benomilo + Mancozed, Benomilo, Carbendazin and distilled water had the least inhibition.

For *F. decemcellulare* Chlorothalonil + Carboxamide and Propiconazole were the best; Benomilo and Carbendazin had a poor performance. *F. mangiferae* showed that the best products for inhibition were Chlorothalonil + Carboxamide, Popiconazole; Mancozed (TR) and Mancozed 70%. The rest of the products did not perform well.

The positive results observed with the use of Propiconazole for *F. circinatum, F. laterirtium* and *F. mangiferae* are similar to those reported by Serrano *et al*. (2014) for *F. circinatum* isolated from dead plants of *Pinus radiata*, where it was efficient. In *F. decemcellulare* he had a regular performance.

The Mancozed fungicide applied alone also exerted good control over the four species, a situation contrary to that referred by Mamza *et al*. (2010) in its evaluation on *F. pallidoroseum*, isolated from *Ricinus communis*. However, when Mancozed 70% was tested together with Benomilo, it had a low response for *F. circinatum* and *F. decemcellulare* and null for *F. lateririum* and *F. mangiferae* where he behaved as the control of distilled water.

The Benomilo applied alone, had a null functioning by not preventing the growth of the mycelium in any of the species tested, a situation close to that observed by Lara *et al*. (2013) for *F. oxysporum* of gerbera with dryer, Mendoza *et al*. (2011) with *F. nivale* and *F. oxysporum* in jicama *Pachyrhizus erosus*, Negrete *et al*. (2007) in *Fusarium* sp., isolated from Heliconia *Heliconia wagneriana* and Jimenez *et al*. (2007) with *Fusarium* sp., by Ginger *Alpinia purpurata* in both cases of radical rot.

The fungicide Carboxamide had variable results, so when it was applied it was only efficient for the species *F. circinatum* and *F. lateritium* but was regular for *F. decemcellulare* and *F. mangiferae*. When it was in combination with Chlorothalonil, its effect was good for *F. circinatum, F. mangiferae* and *F. decemcellulare*, but medium for *F. laterirum*, a situation contrary to that reported by Jimenez *et al*. (2007) where he had good control. Finally, the fungicide Carbendazin did not exert any control over the mycelium of any of the species studied, a situation that is far from that reported by Jiménez *et al*. (2007) and Negrete *et al*. (2007).

Conclusions

Four species of the genus *Fusarium* were isolated from the bark of the rubber sting panel with symptoms of moldy rot. This is a first report that involves this genus. According to the morphological characteristics observed, the Tux-01 isolation corresponded to the species *F. circinatum* Nirenberg & O'Donnell emend. Britz, Coutinho, Wingfield & Marasas. Tux-02 to *F. lateritium* Nees, Tux-03 to *F. decemcellulare* Brick and Tux-04 to *F. mangiferae* Britz, Winfield & Marasas.

In the growth rate, *Fusarium* isolates efficiently developed on culture media ZA, CDA and PDA, but not on FLA where it had a discrete growth. Laboratory pathogenicity tests were negative when no wounds were made on the stem, but positive when wounds were made. Clone IAN-710 was susceptible to the action of these phytopathogens. Field pathogenicity tests were positive for *F. circinatum* and *F. decemcellulare*.

The *in vitro* sensitivity study to fungicides showed great variation, the response depended on the species and fungicide. The need to confirm each described isolation through the use of molecular tools and multigen analysis is evident and indispensable.

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