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Implications of Fusarium spp. in the etiology of stem rot of *Gmelina arborea Roxb (melina)* in the Ecuadorian Humid Tropics.

Implicaciones de Fusarium spp., en la etiología de la pudrición del fuste de *Gmelina arborea Roxb (melina)* en el Trópico Húmedo Ecuatoriano

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ABSTRACT

Gmelina arborea Roxb is a fast-growing forest species adapted to the edaphoclimatic conditions of Ecuador, a quality that has stimulated the establishment of intensive plantations for commercial purposes. A disease with characteristics of vascular wilt and stem rot is killing thousands of trees in the country. *Ceratocystis fimbriata* is listed as the causative agent of the disease, However, the complex symptoms and the frequent isolation of *Fusarium* spp. from diseased trees, make us suspect that this phytopathogen is involved in the pathogenesis of melina. Koch's Postulates were applied at the greenhouse level, and for the effect, 5 treatments based on the inoculation of 17 *G. arborea* plants of 4-month-old per treatment were plated. T1 = *Fusarium* sp.1, T2 = *Fusarium* sp.2, T3 = *Fusarium* sp.3, T4 = *Fusarium* sp.4, and T5 = agar-agar (control). A complete randomized design (CRD) was used and the plants were evaluated 155 days after inoculation. The treatments *Fusarium* sp.1 and *Fusarium* sp.2 caused the largest apparent volumes of necrosis (2.31 cm3 and 2.43 cm3), and generated mild symptoms of disease, however they did not die. These results are considered as a baseline, and would indicate the involvement of *Fusarium* spp. in melina disease, but they are not conclusive.

Keywords: Apparent volume of necrosis, Koch's postulates, pathogenesis.

RESUMEN

Gmelina arborea Roxb es una especie forestal de rápido crecimiento adaptada a las condiciones edafoclimáticas del Ecuador, cualidad que ha estimulado el establecimiento de plantaciones intensivas con fines comerciales. Una enfermedad con características de marchitez vascular y pudrición del fuste está matando miles de árboles en el país. *Ceratocystis fimbriata* está catalogado como el agente causal de la enfermedad, sin embargo, la compleja sintomatología y el frecuente aislamiento de *Fusarium* spp. desde árboles enfermos, hacen sospechar que este fitopatógeno está implicado en la patogénesis de melina. Se aplicaron los Postulados de Koch a nivel de invernadero, y para el efecto se platearon 5 tratamientos basados en la inoculación de 17 plantas de *G. arbórea* de 4 meses de edad, por tratamiento. T1= *Fusarium* sp.1, T2 = *Fusarium* sp.2, T3 = *Fusarium* sp.3, T4 = *Fusarium* sp.4, y T5 = agar-agar (control). Se empleó un diseño completo al azar (DCA) y las plantas se evaluaron a los 155 días después de inoculadas. Los tratamientos *Fusarium* sp.1 y *Fusarium* sp.2 ocasionaron los mayores volúmenes aparentes de necrosis (2.31 cm^3 y 2.43 $cm^{3)}$, y generaron síntomas leves de enfermedad, sin embargo no murieron. Estos resultados se consideran como línea base, e indicaría la implicación de *Fusarium* spp. en la enfermedad de melina, pero no son concluyentes.

Palabras clave: Patogénesis, postulados de Koch, volumen aparente de necrosis.

INTRODUCTION

Gmelina arborea Roxb. (melina), belonging to the Lamiaceae family, native to southwest Asia, is a fast-growing species widely distributed in the Ecuadorian Humid Tropics (THE). It is considered a timber tree valued in the international industry for the production of paper pulp, and also has excellent characteristics for the manufacture of furniture and tertiary products (paper and beams) (Moya, 2004). According to MAGAP (2016) since the introduction of *G. arborea* to Ecuador, it has become an important item for the country's economy, with a significant planted area, where until 2015 there were 11458 hectares, representing 21.9% of the 52395 ha, planted with other economically important forest species (teak, balsa, pine, others) registered in the country.

According to Saltos-Sampedro (2019), in the last five years, a complex and aggressive disease is affecting commercial melina plantations in THE, manifesting itself with a premature and gradual detriment of vigor in the trees, accompanied by discoloration of the leaf system (chlorosis) and stunted growth. It can be observed in some trees that excrete dark brown exudates from the trunk, with a strong odor of decomposing matter, indicating internal rotting of the trunk.

The phytosanitary problem detected in melina seems to be associated with fungal microorganisms, due to the characteristics of the disease and the presence of signs at field level. In this sense, Macías-Moncayo, (2019), through pathogenicity tests demonstrated that the ascomycete fungus *Ceratocystis fimbriata* was the cause of the disease. However, periodic visits to THE plantations show diseased trees with a different symptomatology to the one previously described by Macías-Moncayo, (2019), leading to the suspicion that two diseases are occurring at the same time in *G. arborea* forests and probably caused by different phytopathogens.

This distinctive disease manifests itself with the rotting of the stem, and the consequent death of the standing trees. This rotting generates circular, oval or elongated areas in the bark, acquiring a cracked aspect of dark brown to black color, with a canker typology, whose necrotic area can cover the circumference of the tree. The symptomatology begins with wilting of the leaves and subsequent drying, until they finally fall to the forest floor. Reports in the scientific literature report this type of symptomatology associated with *G. arborea* trees in Costa Rica, where the fungus Deuteromycete *Fusarium* sp., with its sexual phase (ascomycete) *Nectria* sp., has been associated as the cause(s) of the problem (Arguedas, 2004; Murillo-Gamboa *et al.,* 2016).

Concomitant to this information, Saltos-Sampedro (2019), Macías-Moncayo (2019) and Belezaca-Pinargote *et al.,* (data in process of publication) in their research work continuously isolated strains of *Fusarium* spp. from necrotic tissues of diseased melina trees, so their participation in the pathology must be elucidated. Therefore, through this research and using

strains of *Fusarium* spp. previously isolated from diseased melina trees, we sought to determine whether this phytopathogen was capable of causing disease in seedlings according to Koch's postulates, and generating symptomatology similar to that observed in the field.

MATERIALS AND METHODS

Location of experimental site. The present study was conducted in the laboratory of Environmental and Plant Microbiology of the Universidad Técnica Estatal de Quevedo (UTEQ), where the collection of *Fusarium* spp. strains, collected from diseased melina trees, is located.

Activation of *Fusarium* spp. strains were reactivated in Petri dishes containing 10 mL of potato, dextrose, agar (PDA) culture medium plus 0.2 mL of an antibiotic mixture (50 µg/mL penicillin and 25 µg/mL streptomycin), under aseptic conditions, and then incubated for 8 days at 24±2 ^{oC} (Parkinson, 1994, Suryanarayanan, 2013).

Pathogenicity tests. For the pathogenicity tests, 4-month-old melina plants in good health, with a stem diameter at ground level of approximately 3 cm and a height of 60 cm, from a private nursery, were used. For this purpose, the site to be cut was disinfected with cotton moistened with alcohol, and an inclined cut was made with a sterile scalpel, compromising the bark and xylem of the plant. Inside the wound, a colony segment (0.5 cm disk) of the selected phytopathogen was carefully applied and cut with the punch, and once the fungus was inside the plant, the wound was covered with parafilm tape. The control plants were inoculated under the same conditions as above, with the difference that instead of inoculating the pathogen, a segment of agar-agar (innocuous) was applied inside the wound and the wound was closed with parafilm tape (Massimo *et al.,* 2015).

The plants were watered periodically according to their needs. The experiment was established for 155 days (5 months and 4 days), during which time the pertinent observations were made on the health status of each of the inoculated plants, with the purpose of detecting the appearance of symptoms related to stem rot associated with the inoculated phytopathogen. After 155 days of incubation, the plants were dissected by means of longitudinal and transversal cuts to determine and measure (cm) the damage or necrotic lesions in the basal tissues of the plant, both upward and downward, taking as a reference point the central part of the wound made during inoculation. The areas of necrosis were measured in three dimensions (height, width and depth) to estimate the apparent area of necrosis, expressed in cm3 (Zauza *et al.,* 2004).

Treatments and Experimental Design. A completely randomized design (CRD) was used, consisting of five treatments: T1 = melina plants inoculated with *Fusarium* sp.1, T2 = melina plants inoculated with *Fusarium* sp.2, T3 = melina plants inoculated with *Fusarium* sp.3, T4 = melina plants inoculated with *Fusarium* sp.4, T5 = uninoculated melina plants (control). For each treatment, 17 melina plants were used (replicates).

Statistical analysis. The quantitative data obtained were analyzed using descriptive statistical tools: mean, standard deviation, standard error, coefficient of variation, etc. To establish the existence or not of significant statistical differences between treatments, the data were analyzed under the analysis of variance scheme (ANOVA) with a significance level of 95% (*P < 0.05),* after checking the assumptions of normality and homoscedasticity of variances. Subsequently, the LSD (least significant difference) test was applied, with a significance level of 95% (*P < 0.05). The SAS 9.0* statistical package for Windows was used for this purpose.

RESULTS

Apparent volume of necrosis (cm3) generated by Fusarium spp. Significant statistical differences (F=7.73; P=0.000) were detected between the apparent volumes of necrosis generated by the inoculated phytopathogens (treatments) on melina plants. Treatments Fusarium sp.1 and Fusarium sp.2 generated the highest apparent necrosis volumes, with 2.31 cm3 and 2.43 cm3, respectively, and slightly similar to treatment Fusarium sp. 4 with 1.71 cm3, being statistically similar to each other, and different from treatment Fusarium sp.3. However, the control treatment reached the lowest necrosis volume with 0.03 cm3 (Figure 1).

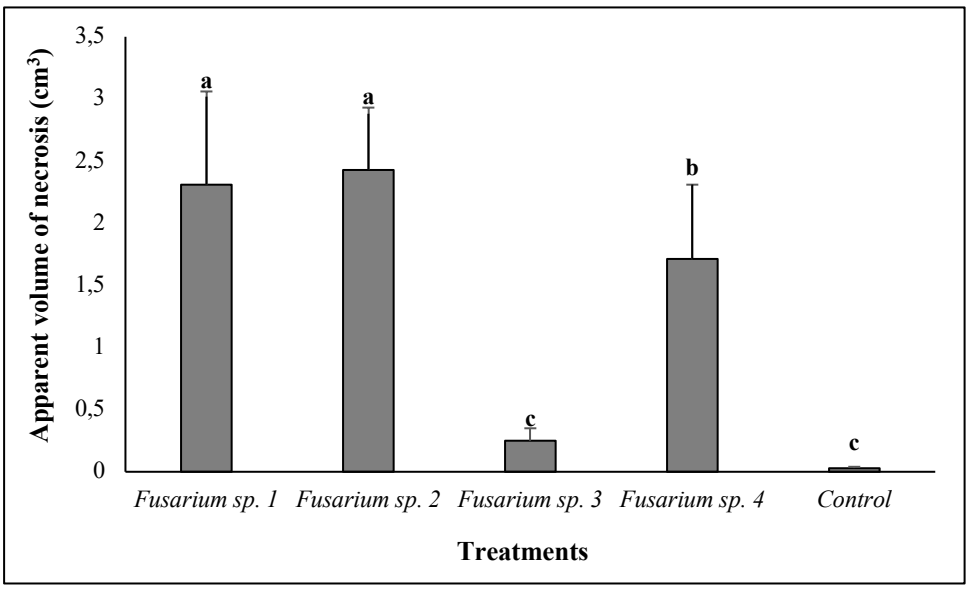

Figure 1 Apparent volume of necrosis generated by phytopathogenic fungi (treatments) inoculated on 4-month-old G. arborea (melina) plants, 155 days after inoculation at greenhouse level. Values correspond to the average apparent volume of necrosis of 17 melina plants, with their respective standard error.

Total length of necrosis (cm) generated by phytopathogens. Figure 2 shows the total length of necrosis caused by phytopathogenic fungi inoculated in melina plants, where significant statistical differences (*F=6*.48; *P=0*.000) were detected between the lengths of necrosis generated by the fungi. The treatments *Fusarium* sp.1, *Fusarium* sp.2, *Fusarium* sp.4, produced the greatest necrosis lengths, with 10.95 cm; 12.91 cm; 9.71 cm, respectively, being statistically similar to each other, but different from the treatments *Fusarium* sp.3 and Control that reached smaller lengths, of 3.76 cm and 1.53 cm, correspondingly.

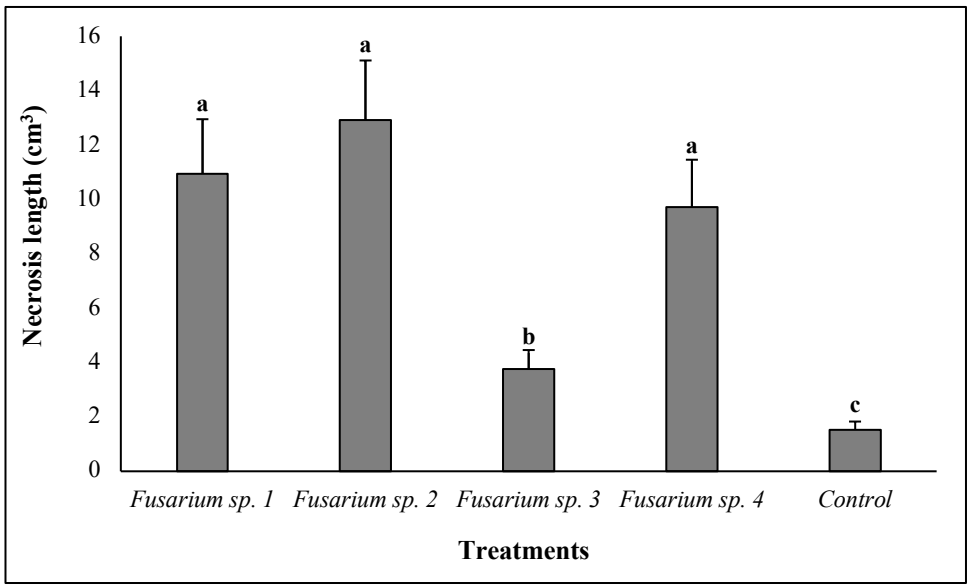

Figure 2 Total length of necrosis, generated by phytopathogenic fungi (treatments) inoculated on 4-month-old *G. arborea* (melina) plants, 155 days after inoculation at greenhouse level. Values correspond to the average apparent volume of necrosis of 17 melina plants, with their respective standard error.

Ascending and descending necrosis length (cm) generated by phytopathogens. Significant statistical differences were detected between the ascending (*F=5*.25; *P=0*.000) and descending (*F=5*.83; *P=0*.000) necrosis lengths caused by the inoculated phytopathogens (treatments) in melina plants. The *Fusarium* sp.2 treatment generated the greatest ascending length of necrosis with 9.08 cm, being statistically superior to the other treatments. While the Control treatment showed the lowest value with 1.05 cm of necrosis length (Table 1).

Table 1.

Ascending and descending length of necrosis (cm) generated by the inoculation of phytopathogenic fungi (treatments) in 4-month-old G. arborea (melina) plants, 155 days after inoculation at greenhouse level. Values correspond to average ascending and descending necrosis lengths of 17 melina plants, with their respective standard error.

Treatments	Ascending length	Standard error	Descending length	Standard error
Fusarium sp.1	6.65 b	1.56	4.30 a	0.69
Fusarium sp.2	9.08 a	1.62	3.83 b	0.73
Fusarium sp.3	2.31 c	0.60	1.44 c	0.33
Fusarium sp.4	6.00 b	1.44	3.71 b	0.75
Control (without	1.05 d	0.26	0.47 d	0.12

Since the introduction of *G. arborea* into THE's production systems in the second half of the 1980s, this species had not presented serious phytosanitary problems. The problems began in the early 2010s, becoming more intense in the following years, despite the fact that the forest species was considered tolerant and/or resistant to the ecological conditions of the region. The increase in phytosanitary problems in melina coincided with the increase in the area planted in intensive monoculture (monospecific plantations), and is probably also associated with genetic materials with good productive potential, but little tolerance to phytopathogens endemic to the complex ecological zones where most melina plantations are established (McKinney *et al.,* 2014).

By means of Koch's postulates it was determined that the apparent volumes of necrosis generated by *Fusarium* spp. in the inoculated plants were variable, this is reflected in the fact that *Fusarium* sp.1, *Fusarium* sp.2, *Fusarium* sp.4, had a similar behavior, although with slight differences, while *Fusarium* sp.3 showed a behavior similar to the control. This could indicate that strains or species of this genus have different levels of pathogenicity for *G. arborea*, a situation that is not surprising since this behavior of the genus *Fusarium* has been reported for several plant species (Shikur *et al.,* 2018). Similar behavior was shown by the fungi in relation to total necrosis length. It is noteworthy that necroses in plants inoculated with *Fusarium* spp. were greater upwards, which would indicate that these fungi prefer to colonize and necrotize vascular tissues from the point of infection (entry) upwards, a situation that was already detected and reported in teak trees at greenhouse level and commercial plantations by Avila-Loor (2016), Belezaca-Pinargote *et al.* (2018), and Solano-Apuntes *et al.* (2019).

The values of apparent necrosis volume and total necrosis length generated by *Fusarium* spp. on inoculated plants resemble those reported by Macias-Moncayo (2019) when he inoculated a strain of *Fusarium* sp. on melina seedlings at greenhouse level and incubated them for 45 days.

The symptoms detected in seedlings inoculated with *Fusarium* sp.1 and *Fusarium* sp.2, begin with a slight chlorosis of the foliar system. However, with the passing of the days the plants did not die. Dissection (longitudinal and transverse cut) allowed us to observe areas of necrosis in the vascular tissues of the inoculated plants. This symptomatological description is quite similar to that detected in young and adult melina trees at field level (Saltos-Sampedro*,* 2019).

The results obtained in this research are not conclusive, but the fact that *Fusarium* sp.1 and *Fusarium* sp.2 treatments generated a greater apparent volume of necrosis stands out, showing a tendency that could indicate their involvement in melina disease, although the results shown here do not yet clearly indicate their role in pathogenesis.

CONCLUSIONS

Plants inoculated with *Fusarium* sp.1 and *Fusarium* sp.2 caused the highest apparent volumes of necrosis, and generated mild disease symptoms, but did not die. These results are considered as a baseline, and would indicate the involvement of *Fusarium* spp. in melina disease, but are not conclusive.

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