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Presence of *Fusarium* spp. complex in diseased trees of *Gmelina arborea* Roxb (melina) in Ecuador

Presencia del complejo Fusarium spp. en árboles enfermos de *Gmelina arborea Roxb* (melina) en Ecuador

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ABSTRACT

The area planted with *Gmelina arborea* (melina) in Ecuador has increased in the last decade, currently this forest species is affected by the disease "vascular wilt and stem rot", which 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, so these results cannot be considered conclusive yet. It is necessary to continue investigating the role of *Fusarium* spp. in the pathogenesis of melina.

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

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RESUMEN

La superficie plantada con Gmelina arbórea (melina) en Ecuador se ha incrementado en la última década, actualmente esta especie forestal es afectada por la enfermedad "marchitez vascular y pudrición del fuste", que 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 cm3 y 2.43 cm3), y generaron síntomas leves de enfermedad, sin embargo no murieron, por lo que estos resultados no se los puede consideran aún concluyentes. Se hace necesario continuar investigando el papel de Fusarium spp. en la patogénesis de melina.

Palabras clave: Patogenicidad, postulados de Koch, volumen aparente de necrosis

1. Introduction

Melina (*Gmelina arborea* Roxb.) is a forest species belonging to the Lamiaceae family, native to southwest Asia, and due to its rapid growth it has been widely planted in the Ecuadorian Humid Tropics (THE). It is considered a timber tree of interest to the national and international industry in the production of paper pulp, and also has excellent characteristics for the manufacture of furniture, tertiary products, pallets, etc. (Moya, 2004). Since the introduction of *G. arborea* to Ecuador, it has become an important item for the country's economy, with a planted area until 2015 of approximately 11458 ha, which represented 21.9% of the 52395 ha, planted with other economically important forest species (teak, balsa, pine, others) registered in the country (MAGAP (2016).

However, in the last five years, a complex and aggressive disease has been affecting commercial plantations of *G. arborea* in Ecuador, manifesting itself with a premature and gradual decline in the vigor of 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 decaying matter, indicating internal rotting of the trunk (Saltos-Sampedro, 2019).

Due to the presence of signs at field level and characteristics of the disease, the phytosanitary problem detected in melina seems to be associated with fungal microorganisms. Studies conducted by Macías-Moncayo (2019) demonstrated the presence and pathogenicity of the ascomycete fungus *Ceratocystis fimbriata*, associating it as the cause of the disease. However, periodic visits to THE plantations report diseased trees with symptoms different from those previously described by Macías-Moncayo (2019), leading to the suspicion that two diseases are occurring at the same time in *G. arborea* forests, probably caused by different phytopathogens (Belezaca-Pinargote *et al.,* 2021).

This distinctive disease manifests itself with rotting of the stem, and the consequent death of the standing trees. The rotting generates circular, oval or elongated areas in the bark, acquiring a cracked appearance of dark brown to black color, with a canker typology, whose necrotic area can cover the circumference of the tree. The symptomatology begins with the wilting of the leaves and subsequent drying, until they finally fall to the ground of the plantation. Scientific literature reports that this type of symptomatology is associated with *G. arborea* trees in Costa Rica, being caused by the fungus Deuteromycete *Fusarium* sp., whose sexual phase is *Nectria* sp. (ascomycete), (Arguedas, 2004; Murillo-Gamboa *et al.,* 2016).

In this sense, Saltos-Sampedro (2019), Macías-Moncayo (2019), and Belezaca-Pinargote *et al.* (2021) in independent research works, constantly isolated *Fusarium* spp. strains from xylem tissues of diseased melina trees, so their participation in the etiology of the disease needs to be clarified. This paper shows the results obtained on the symptomatology, incidence and severity of the disease in melina plantations, *Fusarium* spp. strains isolated and inoculated in seedlings, and their pathogenicity capacity in the plant according to Koch's postulates.

2. Materials and Methods

Location of experimental site. The present study was carried out in the laboratory of Environmental and Plant Microbiology of the State Technical University of Quevedo (UTEQ), where the collection of *Fusarium* spp. strains, collected from diseased melina trees, is located. The field research was carried out in three plantations in the parishes of San Jacinto de Buena Fe (Los Ríos Province) with an age of 4.5 years, Santa María del Toachi and Luz de América (Santo Domingo de los Tsáchilas Province) of 2.3 years and 2.0 years of age, respectively. The

plantations received a very heterogeneous silvicultural management. Weed management was done manually and chemically. The initial density and at the time of the evaluations was different among the plantations (Table 1).

Table 1. *Initial density and density detected at the time of the evaluations in three plantations of G. arborea with different ages.*

No.	Location (Parishes)	Age (years)	Initial density (trees ha-1)	Density at time of evaluation (trees ha-1)
1	San Jacinto de Buena Fe	4.5	1111	953
	Santa María del Toachi	2.3	1111	840
	Light of America	2.0	1111	940

Symptomatological description and incidence and severity of the disease. In each plantation, three rectangular plots of 500 ^{m2} were delimited, within which a tree-by-tree census was carried out, with the purpose of establishing the total number of trees present, the number of trees with disease symptoms, dead trees and apparently healthy trees. The equation [1] used by Belezaca-Pinargote *et al.* (2018) was used for the calculation:

$$\text{Incidencia } (\%) = \frac{\text{N° de árboles enfermos}}{\text{N° de árboles sanos}} * 100 \qquad [1]$$

The estimation of severity was based on the visible morphological characteristics of branches, leaves and stems of diseased trees, and then compared with apparently healthy trees, in addition to a description of the disease symptomatology. For this purpose, an arbitrary scale of five categories proposed by Salas-Rodríguez *et al.* (2016) was applied, as detailed in Table 2.

Table 2. *Arbitrary five-category scale proposed by Salas-Rodríguez et al. (2016) to assess stem rot disease severity in G. arborea.*

Severity (Criterion)	Symptoms
1	Apparently healthy tree, no evidence of visible symptoms.
	Initial yellowing of the crown (evident foliar wilting); the stem may have small necrotic wounds with black exudation in places other than where pruning has occurred; resprouting may begin. Not all symptoms are expressed.
	The tree is visibly diseased. There are canker-like lesions on the bark with signs of rot, bark exposure and bulging, prominent exudation; loss of more than 50% of leaf area in a progressive pattern; regrowth is developed.
	Total affectation of the individual; total absence of foliage; there is evident loss and detachment of branches; resprouts are still observed in some sectors of the trunk; the apparent external rotting reaches 75% of the trunk, where the cancerous zone (canker) is clearly manifested.
5	Completely dry, rotten tree; the wood has completely lost its commercial value.

Isolation of Fusarium spp. strains. 3 trees with disease symptoms were sampled in each plot. The trees were felled at ground level with the assistance of a chainsaw and then transverse and longitudinal cuts were made in the trunk every 50 cm, with the purpose of determining the site of entry of the pathogens and their dissemination within the tissues.

Sections of wood with evidence of necrosis were selected, stored in plastic bags with their respective labeling, and transferred to the Environmental and Plant Microbiology Laboratory of the UTEQ for their respective microbiological analysis. For this purpose, the samples were conditioned as follows:

- **Humid chamber.** Wood samples with necrotic tissues were placed in plastic bags containing moist paper and incubated for 96 hours in conditions of high relative humidity and constant temperature (24 ± 2 °C) at laboratory level. Once

signs of microorganisms (mycelium, fruiting bodies, etc.) growing on the wood were detected, with the assistance of a stereomicroscope, they were transferred to potato, dextrose, agar (PDA) culture media under aseptic conditions.

- **Direct seeding in PDA culture media.** Using a sharp knife, approximately 0.5 x 0.5 cm wood segments were cut from internal necrotic tissues of each diseased tree sampled, and seeded four necrotic wood pieces in five Petri dishes containing 10 mL of PDA culture media + 0.2 mL of an antibiotic mixture (50 µg/mL penicillin and 25 µg/mL streptomycin), (Parkinson, 1994; Massimo *et al.,* 2015; Belezaca-Pinargote *et al.,* 2018), and left to incubate for 96 hours at 24±2 ^{oC}. After this time, the fungi developed in the culture medium were identified with the help of dichotomous taxonomic keys, based on morphology (Von Arx, 1981; Barnett & Hunter, 1987).

Activation of previously isolated *Fusarium* spp. strains. 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).

Koch's postulates. For this purpose, 4-month-old melina plants in good health, with a stem diameter at ground level of approximately 3 cm and 60 cm in height, from a private nursery, were used. The site to be inoculated was disinfected with alcohol moistened cotton and the bark and xylem of the plant were compromised by means of an inclined cut with a sterile scalpel. A colony segment (0.5 cm disk) of the selected phytopathogen was carefully applied inside the wound, and once the fungus was inside the plant, the wound was covered with parafilm tape. 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 requirements. The experiment was established for 155 days (5 months and 4 days), during which time observations were made on the health status of the inoculated plants, with the purpose of detecting the appearance of symptoms related to stem rot disease, associated with each inoculated phytopathogen. At the end of the study, the plants were dissected through transverse and longitudinal cuts, with the purpose of estimating the damage or necrotic lesions in the bascular tissues of each plant, both upward and downward, taking the inoculation site as a reference point.

Necrotic lesions 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.

3. Results

Symptomatology of the disease. Trees of *G. arborea* in early stages of the disease show a slight chlorosis, and loss of turgor in leaves at the ends of upper branches, with wilting characteristics. Due to vascular blockage and gradual disease progression, growth apices dry out and branches progressively die. In diseased trees, vigor decreases significantly compared to healthy neighboring trees, mainly due to wilting of the photosynthetic area. Once the leaf area is compromised, as a survival mechanism, diseased trees emit numerous epicormic shoots on the stem, stimulated by the need for photosynthesis. However, as vascular plugging continues to impede the ebb and flow of nutritional substances, the tree eventually dies (Figure 1). In diseased trees it is common to observe the presence of dark-colored fluids, released from wounds of anthropogenic origin (pruning wounds, thinning, natural wounds, etc.), (Figure 2). It should be noted that in diseased trees the presence of bark and/or wood boring insects is generally not observed.

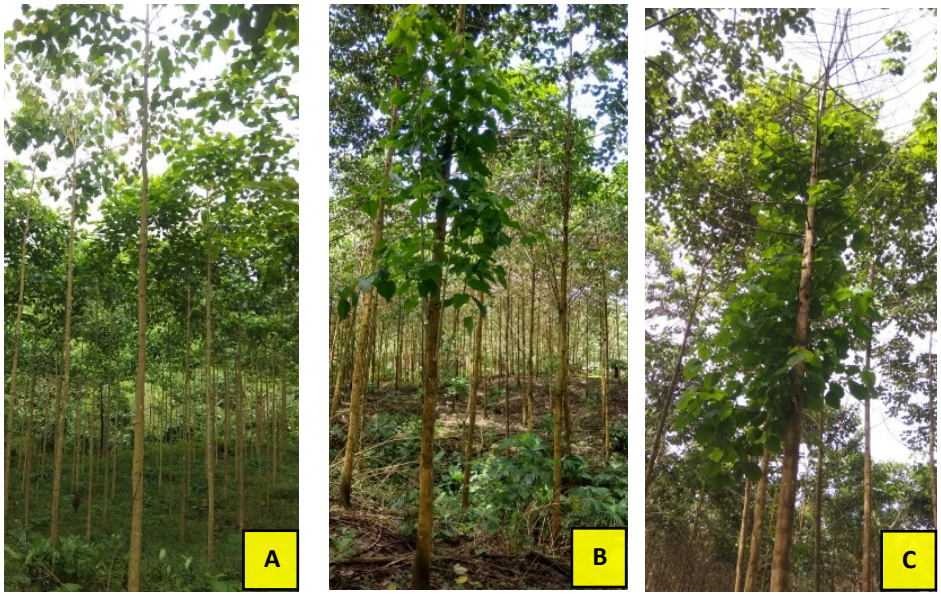

Figure 1. Evolution of vascular wilt disease and stem rot in melina trees: A = First symptoms characterized by leaf chlorosis (wilting) due to vascular plugging. B = Generation of multiple epicormic shoots on the stem. C = Death of the foliar system of the tree.

Since the introduction of *G. arborea* in THE production systems in the mid-1980s, the species had not presented complex phytosanitary problems, but in the last decade due to the increase in planted area, phytosanitary problems have become recurrent as mentioned by Saltos-Sampedro (2019) and Macías-Moncayo (2019). The appearance of diseases in monospecific plantations is related to massification, the increase in planted area, the susceptibility of the species and time, factors that, from the point of view of Forest Pathology, predispose trees to disease (Haas *et al.,* 2011; Bostock *et al.,* 2014; Hughes *et al.,* 2015).

The vascular wilt and stem rot disease in melina, presents a complex symptomatology, similar to that reported in other economically important forest species in the region, such as *Schizolobium parahybum* (Geldenhuis *et al.,* 2004; Belezaca-Pinargote *et al.,* 2011), *Acrocarpus fraxinifolius* (Belezaca-Pinargote *et al.,* 2012) that destroyed entire plantations between the 1990s and 2000s, and *Tectona grandis* (Belezaca-Pinargote *et al.,* 2018; Vera *et al.,* 2019; Belezaca-Pinargote *et al.,* 2020), whose disease appeared early in the past decade and continues to eliminate thousands of trees in THE.

The behavior of *G. arborea trees against the* disease is not uniform, generally the manifestation of the initial symptoms are difficult to detect, since chlorosis and

loss of turgor occurs when the xylem and bascular tissues of the trees are irremediably compromised and necrotic, both radially and tangentially. Defoliation, death of growth buds and drying of branches are observed in advanced stages of the disease. The presence of natural and/or anthropogenic wounds on the trunk is common, with abundant exudation of dark brown fluids, with a strong odor of decomposing organic matter.

Incidence and severity of the disease. In the 2-year-old plantation, an average of 10 diseased trees and 2 dead trees per plot (500 ^{m2}) were detected, which allowed inferring the existence of 215 diseased trees and 45 dead trees per ha-1. In the plots of the 2.3 year old plantation, an average of 3 diseased trees and no dead trees were found, resulting in 60 diseased trees and zero dead trees per ha-1. While in the 4-year plantation, 4 diseased trees and 2 dead trees were found on average per plot, resulting in 80 diseased trees and 40 dead trees per ha-1. These results show that disease incidence was 24.1%, 7.1%, and 21.3% for the 2-, 2.3-, and 4-year-old melina plantations, respectively (Table 3).

Table 3. *Number of apparently healthy, diseased and dead trees per ha-1 and incidence of vascular wilt disease stem rot in three plantations of G. arborea of different ages in the Ecuadorian Humid Tropics. Values represent the average of three replicates, with their respective standard error.*

Plantation (age)	Trees ha-1 (Density)	Apparently healthy trees ha-1	Diseased trees ha-1	Dead trees ha-1	Incidence (%) *
2 years	1080	820 ± 0.67	215 ± 0.25	45 ± 0.80	24.1 ± 1.15
2.3 years	840	780 ± 0.75	60 ± 0.61	0,0 ± 0.00	7.1 ± 0.95
4 years	940	740 ± 0.90	140 ± 0.40	60 ± 0.28	21.3 ± 0.87

Most of the diseased trees in the 2-year-old plantation were classified in scales 2 and 4, which indicates that 9.52% of the trees were found with the presence of the disease, in a state of medium progress, and 7.14% were dead (scale 5). While in the 2.3 year old plantation, diseased trees were located in scale 2 with 7.14%. In the 4-year-old plantation, trees with different levels of disease were detected

in scales 2, 3, 4 and 5, with 8.51%, 4.25%, 2.13% and 6.38%, respectively (Table 4).

Table 4*. Severity of vascular wilt disease and stem rot per ha-1, in plantations of G. arborea of 2, 2.3, and 5 years of age.*

Plantation (age)	Trees ha-1 (Density)	No. trees per scale *			
		1			5
2 years	1080	820	45		45
2.3 years	840	780	0	0	0
4 years	940	740			

It is worth noting the resilience of several melina trees, which at the time of evaluation do not show any visible symptoms of the disease; however, when they are cut transversally and longitudinally along the trunk, the internal tissues are completely necrotic in all directions, which makes it difficult to identify trees in the initial stages of the disease. On the other hand, field observations show the existence of trees to which the disease does not seem to cause major damage, which leads to the suspicion of the presence of individuals with acceptable levels of tolerance/resistance to the disease. This behavior would be due to the genetic variability of individuals obtained from freely pollinated seeds (Bräutigam *et al.,* 2013; Inza *et al.,* 2018), which can be exploited in future genetic improvement programs of the species.

The incidence of the disease in the three plantations (24.1%, 7.1%, and 21.3%), could be considered high, and a warning about the damage that can be generated to the national melina industry, if adequate strategies for prevention and management of the disease are not considered. Although diseased trees were found at all levels of the proposed scale, most of them were detected in the initial stage of the disease, but it is a matter of time before the symptomatology progresses and the trees move to higher levels of the scale.

Isolation of *Fusarium* spp. After analyzing the wood sections with the aid of a stereomicroscope, the presence of yellowish-white cottony colonies of fungal appearance was determined. In addition, sections of necrotic wood seeded in Petri dishes with PDA culture medium, after 96 hours of incubation, resulted in the growth of white cottony colonies. Observations under the microscope identified that the colonies corresponded to the fungus *Fusarium (*Figure 2).

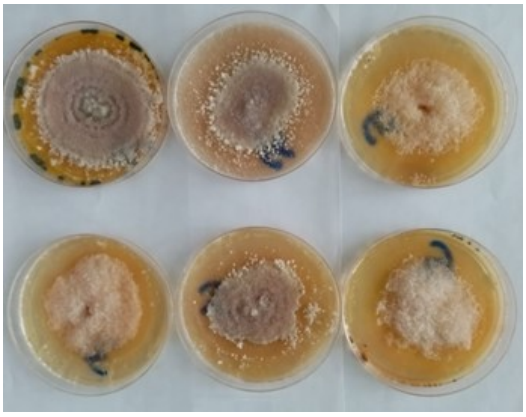

Figure 1. Colonies of *Fusarium* spp. obtained from diseased trees of *G. arborea*, growing on PDA culture medium.

Apparent volume of necrosis (cm3). 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. The *Fusarium* sp. 1 and *Fusarium* sp. 2 treatments generated the highest apparent necrosis volumes, with 2.31 cm3 and 2.43 cm3, respectively, and slightly similar to the *Fusarium* sp. 4 treatment with 1.71 cm3, being statistically similar to each other, and different from the *Fusarium* sp. 3 treatment. However, the control treatment reached the lowest necrosis volume with 0.03 cm3 (Figure 3).

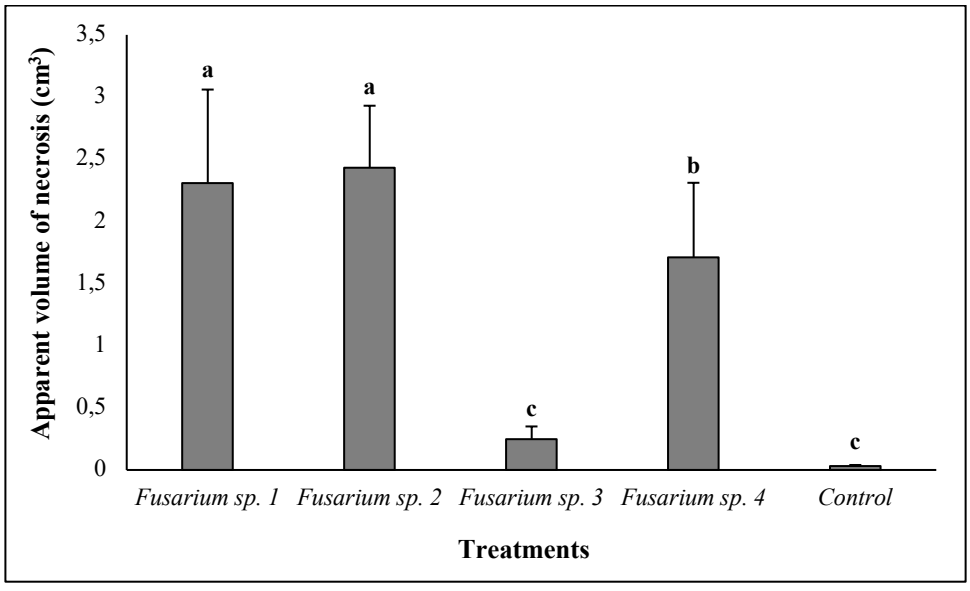

Figure 3. *Apparent volume of necrosis generated by phytopathogenic fungi (treatments) inoculated on 4-month-old G. arborea (melina) plants, 155 days after*

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 4 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.

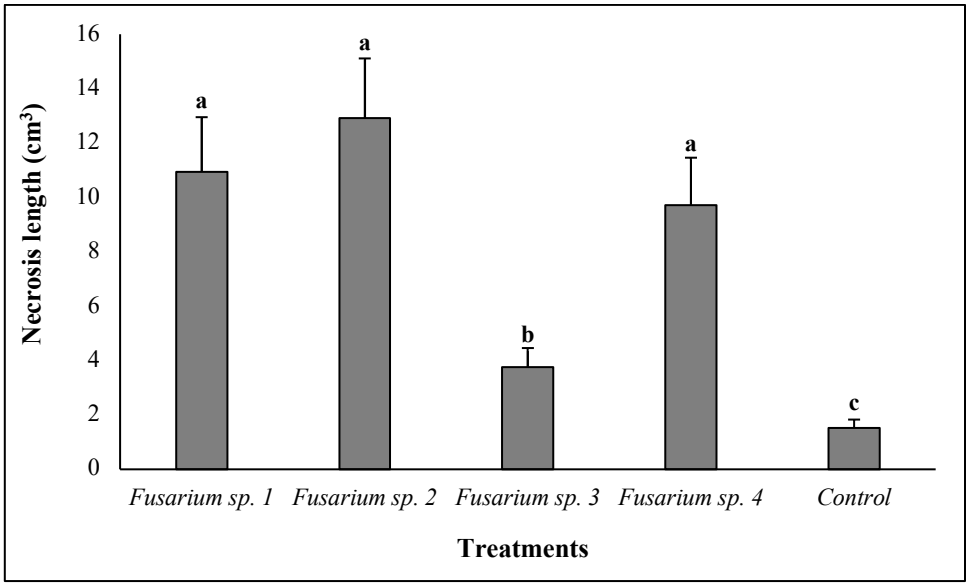

Figure 4. Total length of necrosis, generated by phytopathogenic fungi (treatments) inoculated on 4-month-old *G. arborea* (melina) plants, 155 days after inoculation at greenhouse level. Values correspond to the average apparent volume of necrosis of 17 melina plants, with their respective standard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

Ascending and descending necrosis length (cm). 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 5).

Table 5. *Ascending and descending length of necrosis (cm) generated by the inoculation of phytopathogenic fungi (treatments) in 4-month-old G. arborea (melina) plants, at 155 days of 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	6.65 b	1.56	4.30 a	0.69
Fusarium	9.08 a	1.62	3.83 b	0.73
Fusarium	2.31 c	0.60	1.44 c	0.33
Fusarium	6.00 b	1.44	3.71 b	0.75
Control (without inoculation)	1.05 d	0.26	0.47 d	0.12

These results could indicate that the inoculated pathogen strains or species have different levels of pathogenicity for *G. arborea*, a situation that is not surprising since this behavior of the *Fusarium* genus 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), Solano-Apuntes *et al.* (2019), and Belezaca *et al.,* 2020.

Additionally, the values of apparent necrosis volume and total necrosis length generated by *Fusarium* spp. in inoculated plants resemble those reported by Macías-Moncayo (2019) when he inoculated a strain of *Fusarium* sp. in 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 reported in young and adult melina trees at field level by Saltos-Sampedro (2019) and Belezaca-Pinargote *et al.,* 2021.

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.

4. 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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