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Colonization of *Fusarium oxysporum* transformed with the red fluorescence protein gene (tdTomato) mediated by *Agrobacterium tumefaciens* in roots of two avocado cultivars

Colonização de *Fusarium oxysporum* transformada com o gene da proteína fluorescente vermelha (tdTomato) mediada por *Agrobacterium tumefaciens* em raízes de dois cultivares de abacate

Colonización de *Fusarium oxysporum* transformado con el gen de la proteína de fluorescencia roja (tdTomato) mediado por *Agrobacterium tumefaciens* en raíces de dos cultivares de aguacate

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Abstract
Avocado (*Persea americana*) is a fruit crop of economic importance in Ecuador. Currently, a low incidence of *Phytophthora cinnamomi* has been reported, however, there are other soil pathogens that can affect this crop, even at the initial stages of plant multiplication (nursery), for this reason the use of rootstocks that tolerate these biotic adversities is recommended. In this research, the fungus *Fusarium oxysporum* was isolated from roots of nursery seedlings with symptoms of necrosis. In addition, an isolate of this pathogenic fungus modified with a strain of *Agrobacterium tumefaciens* was used to determine the infection of *F. oxysporum* in the roots of the Fuerte (commercial) and Criollo (local) cultivars. The results allowed to infer that the cultivar Criollo presented a greater tolerance to *F. oxysporum* than the cultivar Fuerte, which corroborates its use as a rootstock for commercial avocado varieties. Furthermore, to our knowledge, this is the first report of *F. oxysporum* affecting avocado nursery seedlings in Ecuador.
Keywords: Agricultural biotechnology; Criollo; Fuerte; Hygromycin B; Fluorescence microscopy; Rootstock.

Resumo
O abacate (*Persea americana*) é uma árvore frutífera de importância econômica no Equador. Atualmente, tem sido relatada uma baixa incidência de *Phytophthora cinnamomi*, entretanto, existem outros patógenos de solo que afetam esta cultura, mesmo nos estágios iniciais de multiplicação das plantas, por isso é recomendado o uso de porta-enxertos que toleram essas adversidades bióticas. Nesta pesquisa, o fungo *Fusarium oxysporum* foi isolado de mudas de viveiro de raízes com sintomas de necrose. Além disso, um isolado deste fungo patogênico modificado com uma cepa de *Agrobacterium tumefaciens* foi usado para determinar a infecção de F. oxysporum nas raízes dos cultivares Fuerte e Criollo. Os resultados permitem inferir que o cultivar Crioulo apresentou maior tolerância ao *F. oxysporum*, o que

corrobora seu uso como porta-enxerto para variedades comerciais de abacate. Além disso, até onde sabemos, este é o primeiro relato de *F. oxysporum* afetando mudas de abacate.
Palavras-chave: Biotecnologia agrícola; Criollo; Fuerte; Higromicina B; Microscopia de fluorescência; Porta-enxerto.

Resumen
El aguacate (*Persea americana*) es un frutal de importancia económica en el Ecuador. Actualmente se ha reportado una baja incidencia de *Phytophthora cinnamomi*, sin embargo, existen otros patógenos del suelo que afectan a este cultivo, incluso en etapas iniciales de multiplicación de la planta, por esta razón el uso de portainjertos que toleren estas adversidades bióticas es recomendado. En esta investigación, se aisló el hongo *Fusarium oxysporum* de plántulas de vivero de raíces con sintomatología de necrosis. Además, se utilizó un aislado de este hongo patógeno modificada con una cepa de *Agrobacterium tumefaciens* para determinar la infección de *F. oxysporum* en las raíces de los cultivares Fuerte y Criollo. Los resultados permiten inferir que el cultivar Criollo presentó una mayor tolerancia a *F. oxysporum*, con lo cual se corrobora su uso como portainjerto para variedades comerciales de aguacate. Además, de acuerdo a nuestro conocimiento, este es el primer reporte de *F. oxysporum* afectando a plántulas de aguacate.
Palabras clave: Biotecnología agrícola; Criollo; Fuerte; Higromicina B; Microscopía de flourescencia; Portainjerto.

1. Introduction

Avocado (*Persea americana*) has become a fruit crop with great commercial demand in Ecuador and internationally due to its good taste and nutritional atributes (Viera et al., 2016).

Within the genus *Persea*, three races have been identified: Mexican (*P. americana* variety drymifolia), Guatemalan (*P. americana* variety guatemalensis) and Antillean (*P. americana* variety americana) (Álvarez et al., 2015). In Latin American, most of the cultivated avocados are hybrid varieties that contain genetic material from all three races. One of these is the called Fuerte which is the most important commercial cultivar in Ecuador; however, there are local materials known as Criollos which are frequently used as rootstock for commercial varieties (Viera et al., 2017).

Annually, avocado wilt caused by pathogens leads to losses of between 20% and 40% (Granada et al., 2020). Symptoms observed in young plants are lack of development, loss of brightness and vigor, yellowing of the leaves and generalized wilting, and as the infection progresses, the plant withers, loses leaves and eventually dies (Ramírez et al., 2014). While in the field, the affected trees lose their vigor due to the rotting of the absorbent and secondary roots (Ramírez and Morales, 2020). The main causal agent of avocado root rot is *Phytophthora cinnamomi* Rands, but in Ecuador the incidence of this oomycete is low (Toapanta-Gallegos et al., 2017). On the other hand, it has been reported *Fusarium* sp. in avocados causes wilting symptoms in the United States and Colombia (Ramírez et al., 2014).

Understanding the infection process of phytopathogenic fungi is important. An alternative is the use of genetic engineering tools such as transformation (Dunn et al., 2013). The expression of fluorescence genes in phytopathogenic fungi has allowed a deeper understanding of plant-pathogen interactions (Caasi et al., 2010; Dunn et al., 2013). This study determined the colonization of *F. oxysporum* in avocado roots of Criollo and Fuerte cultivars by the use of the pathogen transformed with the red fluorescence protein gene (tdTomato).

2. Methodology

2.1 Isolation of the pathogen and Koch's postulates.

F. oxysporum was obtained from avocado seedlings showing wilting symptoms in the nurseries of the Tumbaco Experimental Farm of the National Institute of Agriculture Research (INIAP). The pathogen was isolated in the Laboratory of Microbology of the University of the Armed Forces (ESPE) and was coded as UFA0019. Root cuts of 1 cm length with necrosis symptoms were placed in Petri dishes containing Potato Dextrose Agar (PDA) medium supplemented with 100 ppm of chloramphenicol antibiotic, and they were placed in the incubator at a temperature of 25 °C for 7 days. The taxonomic keys

of Barnett and Hunter (1998) were used for the morphological identification of the isolate. To verify the pathogenicity of the isolate, Koch´s postulates (Tronsmo et al., 2020) were carried out. Three healthy seedlings of cultivar Fuerte were inoculated, seedlings were sown in 2.2-liter bags with a substrate formed by black soil and pomine, in a 2: 1 ratio. A non-inoculated seedling kept under the same conditions was used as a control. For the preparation of the inoculum, three fragments of the PDA (1 cm^2) medium with mycelium were taken and multiplied in oat substrate (300 g) and incubated at 25 °C in the dark for 15 days (Bruna, 1991). The inoculum was mixed with the substrate in a 1: 3 ratio, then the avocado seedlings were transplanted. After 90 days, when seedlings began to show the first symptoms of wilting (leaf decay), they were removed from the substrate and roots showing symptoms of necrosis were selected and the pathogen was re-isolated.

2.2 Molecular identification of the pathogen

Mycelium from a monosporic culture was placed in test tubes with peptone liquid medium (Difco Peptone Water) and were incubated at 25 °C for four days. DNA was extracted following the protocol of Weising (1995) and an amplification of the internal transcribed spacer (ITS) was performed with the specific primers ITS4 (5'-TCCTCCGCTTATTGATATGC-3'), and ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') (White et al., 1990). The products of the polymerase chain reaction (PCR) were sequenced and the result was compared with the databases of the National Center for Biotechnology Information (NCBI). Multiple alignment was done with MUSCLE (Edgar, 2004) within MegaX (Smith et al., 2009).

2.3 Sampling and germination of avocado seeds

Avocado fruits of the Criollo and Fuerte cultivars were harvested form the orchards of the Tumbaco Experimental Farm. Seeds were washed with running water and the mesocarp (pulp) was removed. They were dried, the seed coat was removed and were disinfected using Carboxim (2 g/L) (Molano, 2007). Finally, three toothpicks were placed on top of the seed and kept on a glass with 50% volume of water. In a period of eight weeks, the seeds produced roots.

2.4 Transformation of *Agrobacterium tumefaciens*

Agrobacterium tumefaciens strain AGL1 genetically modified with plasmid pBHt2-tdTom (Caasi et al., 2010) was donated by the Department of Entomology and Phytopathology of Oklahoma State University in 2014. The strains were reactivated in *Agrobacterium* minimal medium (AMM) (Hooykaas et al., 1979) supplemented with 50 µg/mL of carbenicillin and kanamycin to maintain the plasmid. The presence of the tdTom gene was confirmed in the *Agrobacterium* with a colony PCR, using the specific primers ATGGTGAGCAAGGGCGAGGAGG (forward) and TTACTTGTACAGCTCGTCCATG (reverse) (Shaner et al., 2004).

2.5 Determination of the minimum inhibitory concentration (MCI)

The minimum inhibitory concentration test was carried out using a modification of the protocol reported by Cruz-Martín et al. (2001). A hygromycin B antibiotic stock solution was prepared with a concentration of 50 mg/mL. The antibiotic was added to the yeast extract, peptone and glucose medium (YPS) at concentrations of 50, 100, 150 and 200 µg/mL. Then a 1 cm^2 of PDA medium with the isolated pathogenic fungus was taken and placed in the center of the Petri dish with the antibiotic. A treatment without antibiotics was used as a control. The concentration at which the growth of *F. oxysporum* was completely inhibited was taken as the MCI.

2.6 Agrobacterium-mediated transformation of *Fusarium oxysporum*

From five-day-old *F. oxysporum* colony incubated at 25 °C in PDA, fungal spore solutions were obtained and adjusted to a concentration between 2×10^4 and 2×10^6 CFU / mL (Mullins et al., 2001). In addition, two 4 mm diameter mycelium fragments of PDA were placed in 1 mL of liquid inducing minimal medium (IMM) (Bundock et al., 1995) with three 3 mm glass balls and the mycelium was broken using a homogenizer (Biospec Product, Minibead beater).

The viability of the spore solution and mycelium fragments was checked, taking 50 µL and dispersing them in PDA. The spores that generated colonies were considered viable and were used for the transformation process.

The protocol of Mullins et al. (2001) was used with the following modifications: to induce the virulence, a colony of *A. tumefaciens* AGL1 was used with the plasmid pBHt2-tdTom grown in the AMM medium for three days and transferred to AMM medium supplemented with 50 µg/mL of carbeniline and kanamycin). It was incubated for three days at 28 ºC with shaking at 150 rpm. Subsequently, AGL1 cells were re-suspended in IMM medium supplemented with 200 µM of acetosyringone, 50 µg/mL of carbeniline and 50 µg/mL of kanamycin. The optical density was adjusted to 0.2 on the spectrophotometer (Thermo Scientific, Genesys UV-Vis) with a wavelength of 600 nm. It was incubated at 28 °C for 10 hours with shaking at 105 rpm. The pellet was collected by centrifugation and re-suspended in IMM at the mentioned optical density.

A co-culture was carried out with the suspension of the induced *A. tumefaciens* and the solution of spores and mycelium fragments of *F. oxysporum* (volume 1: 1). 200 µL of the suspension was placed on the surface of a nitrocellulose membrane in IMM medium supplemented with 50 µg/mL carbenicillin and kanamycin. Afterwards, it was incubated at 25 °C for 72 hours until colonies of the fungus appeared.

The nitrocellulose membranes were transferred to YPS medium supplemented with 150 µg/mL of hygromycin B, 200 µg/mL of cephatoxin and 100 µg/mL of timentin. Then, it was incubated at 25 °C in the dark for 14 days, until the formation of colonies.

The presence of the tdTomato gene was macroscopically verified in a dark chamber with UV light, observing the YPS Petri dishes with growth. Once the transformed fungi were obtained, they were cultivated in selective medium with hygromycin B to purify them and then subcultured in non-selective medium to check the stability of the fluorescence gene after transformation. To maintain the transformants, they were cultivated in selective medium and stored in a deep freezer (Termo Scientific) at -80 °C.

2.7 Evaluation of the colonization of the transformed fungus in avocado roots

A piece of PDA (2 mm^2) with fluorescent mycelium was taken with a sterile syringe and placed next to wounds of 0.5 cm long made in the germinated avocado roots. Germinated seeds were placed in humid chambers at 20 °C and the roots were observed in the fluorescence microscope (Olympus, IX53) after 30 days after inoculation, following the protocol of Flores et al. (2015).

For the evaluation of the root colonization by the transformed fungus, a qualitative scale was used where 0 = no colonization, 1 = presence of mycelium colonizing around the root, 2 = presence of mycelium penetrating the root cut.

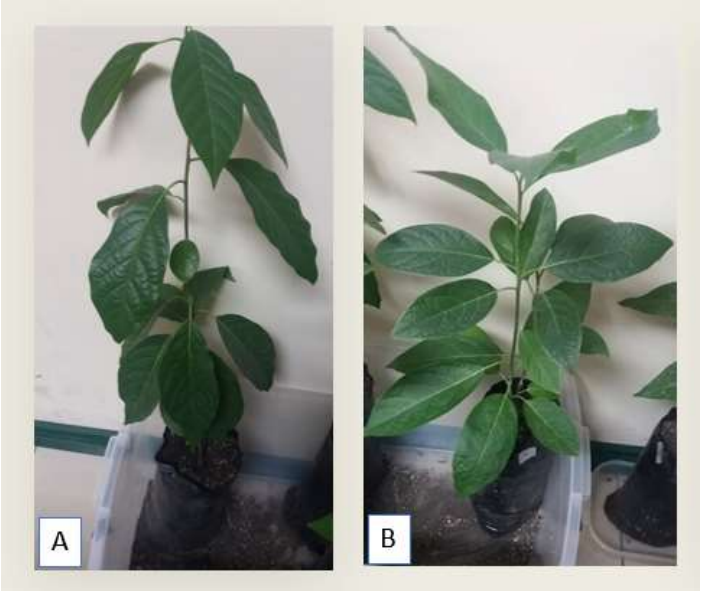
A non-parametric Kruskal Wallis statistical test was performed with a confidence level of 5% ($\alpha = 0.05$) to determine statistical differences between cultivars in the evaluation of the root colonization. Three replicates per cultivar were evaluated and there was a non-inoculated control.

3. Results

3.1 Koch´s postulates

After 90 days of the inoculation of *F. oxysporum* in the avocado seedlings, noticeable symptoms of decay (Figure 1) and necrosis were observed in the main and secondary roots (Figure 2).

Figure 1. A) Avocado seedling inoculated with *Fusarium oxysporum* and B) Control seedling (without inoculation).

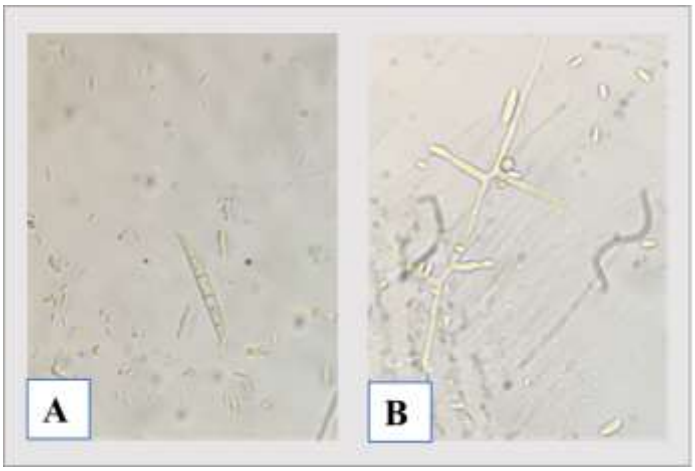


Source: Authors (2021).

Figure 2. Avocado roots showing symptoms of necrosis at 90 days after inoculation with *Fusarium oxysporum*. The inoculated pathogen was reisolated from the roots with necrosis symptoms, obtaining colonies of the fungus with a pink coloration. The fungus was morphologically identified as *F. oxysporum* using the taxonomic keys of Barnett and Hunter (1998). Macroconidia of 5 septa, monofilial chlamydiospores and microconidia were observed (Figure 3).

Source: Authors (2021).

Figure 3. Macro and microconidia of *Fusarium oxysporum* (40 X). A) Macroconidia of 5 septa. B) Chlamydiospora monofilial and microconidia.

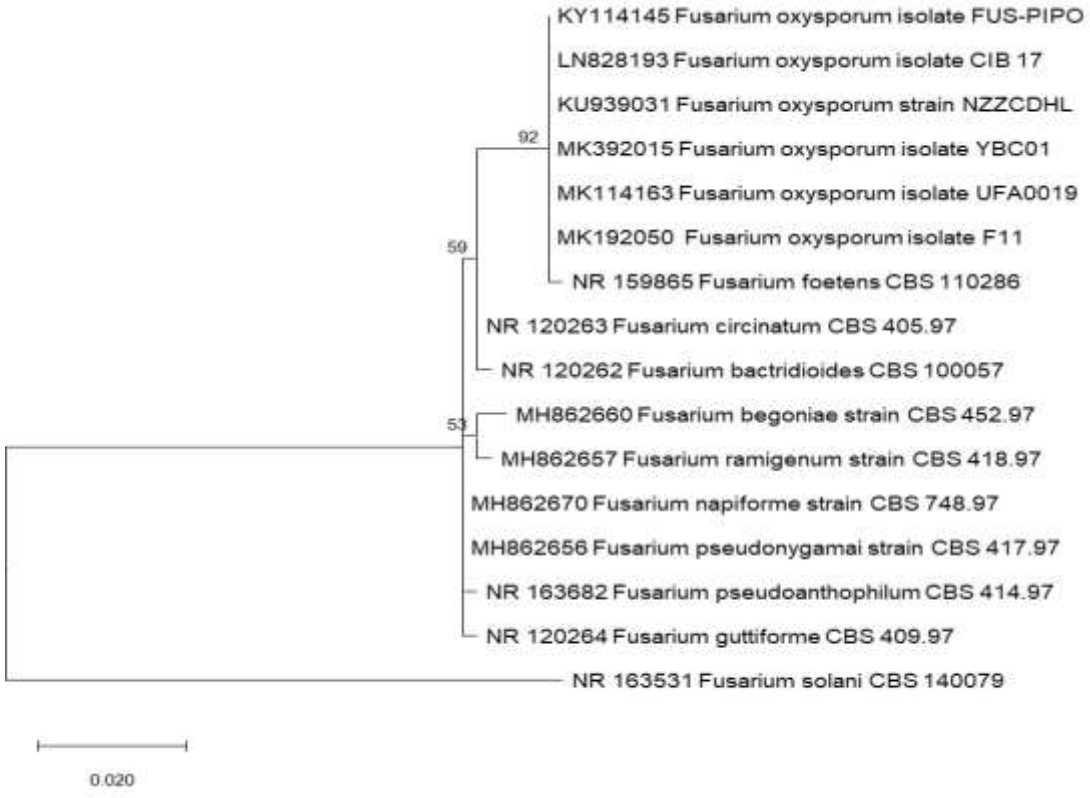


Source: Authors (2021).

3.2 Molecular identification of the pathogen

The sequence obtained was compared with the sequences of the NCBI databases, obtaining 100% identity with sequences from 89 different isolates of *F. oxysporum*, including FUS-PIPO (Argentina), CIB 17 (Colombia), NZZCDHL (China), YBC01 (China) and F11 (India), they were used for the construction of the phylogenetic tree (Figure 4). The sequence of the isolate UFA0019 was stored in GenBank with the accession number MK114163.

Figure 4. Phylogenetic tree of Fusarium isolates from the NCBI base.

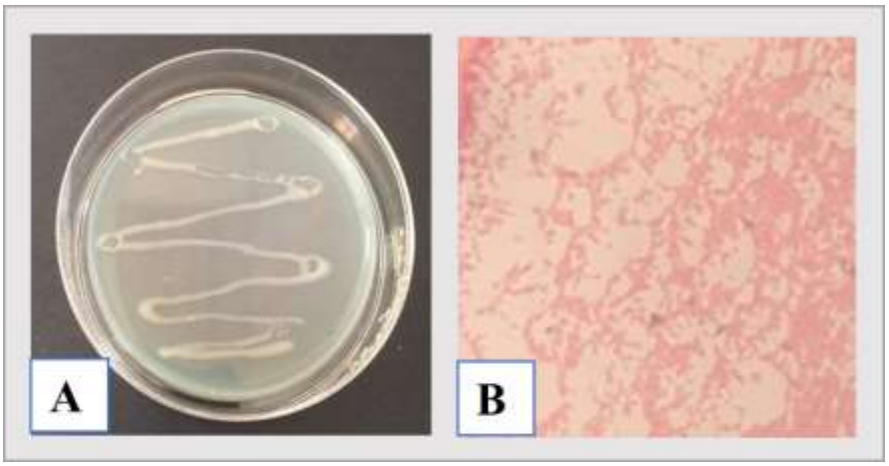


Source: Authors (2021).

3.3 Agrobacterium tumefaciens AGL1

In the AMM medium, at the macroscopic level, the bacteria showed white color, irregular edges and creamy consistency. At the microscopic level, a Gram stain was carried out where the bacteria showed a bacillus shape and pink color which are characteristic of Gram negative organisms (Figure 5).

Figure 5. A) Culture of *Agrobacterium tumefaciens* strain AGL1 in AMM medium. B) Differential Gram staining at 100X.



Source: Authors (2021).

It was observed that the colony-PCR product had a size of 1500 bp, confirming the presence of the tdTomato gene in the bacteria. Furthermore, the presence of the tdTomato gene was verified visually under UV light.

3.4 MCI of hygromycin B for *F. oxysporum*

With respect to the determination of the MCI of hygromycin B for *F. oxysporum*, it was observed that in the concentration of 50 µg/mL there was a growth similar to that of control without antibiotic, at 100 µg/mL there was less growth of the fungus than the control, whereas at 150 and 200 µg/mL there was no growth. The growth of *F. oxysporum* observed at concentrations of 50 µg/mL and 100 µg/mL of hygromycin, therefore its MCI was determined at 150 µg/mL of the antibiotic.

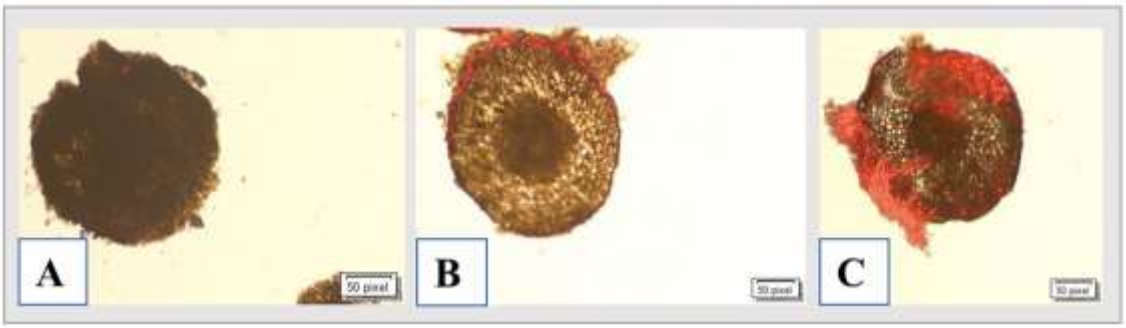
3.5 Evaluation of the root colonization using the transformed fungus

Colonization in cultivars was observed at 30 days (Figure 6) through cross sections of the roots inoculated with the transformed fungus. An average value of 0.62 for the Criollo cultivar and 1.04 for Fuerte with the scale used.

The non-parametric statistical test of Kruskal Wallis ($\alpha = 0.05$) determined that there were statistical differences ($p = 0.0494$) between cultivars in the percentage of root colonization.

In terms of percentage, the cultivar Criollo showed 49% non-colonization in the roots, 37% presence of mycelium around the root and 14% presence of mycelium in the root cut. While the cultivar Fuerte showed that 16% of the inoculated roots did not show colonization, 62% presence of mycelium around the root and 21% presence of mycelium in the root cut.

Figure 6. Images about avocado root colonization. A) Scale 0 = no colonization. B) Scale 1 = presence of hyphae around the root (cultivar Criollo). C) Scale 2 = presence of hyphae in the root cut (cultivar Fuerte).



Source: Authors (2021).

4. Discussion

4.1 Koch´s postulates

Among the signs associated with avocado wilt is the lack of development, decay and yellowing of the leaves (Ramírez et al., 2014; Ramírez and Morales, 2020). In our study, a noticeable decay of avocado leaves was observed 90 days after inoculation with *F. oxysporum* but foliar yellowing was not observed because it requires a longer time to express this sympton (Olalde et al., 2020). However, the pathogen infection was confirmed by the presence of necrosis symptoms in the roots of the inoculated seedlings and the re-isolation of the pathogen.

Macroscopically, the re-isolated colonies showed a cottony appearance and a whitish-pink tonality which is characteristic of *F. oxysporum* (Olalde, et al., 2020). Microscopically, the isolates produced conidia with morphology congruent with the genus *Fusarium* (Barnett & Hunter, 1998).

4.2 Characterization of *Agrobacterium tumefaciens* and confirmation of the presence of the tdTomato gene in the plasmid

Kado et al. (1972) described A. tumefaciens colonies of bone white color and circular in shape and microscopically present as a Gram negative bacillus. In their research, the Agrobacterium strain used presented the same characteristics as mentioned by the author.

To verify the presence of the tdTomato gene, a colony-PCR was performed, a method that is used for rapid scanning from colonies of yeast or bacteria that have grown in a selective medium after a transformation process to verify the presence of the genes of interest (Bergkessel and Guthrie, 2013). The amplified gene had an approximate size of 1500 bp, value congruent with the expected size of the amplicon generated by specific primers for tdTomato (1431 bp) (Shaner et al, 2004).

4.3 Determination of the minimum inhibitory concentration of *Fusarium* for hygromycin B

In order to carry out the genetic transformation process, selection agents are needed that are able to discriminate between the transformed and non-transformed strains. One of the most widely used methods is exposing organisms to antibiotics; using this, it is intended to eliminate non-transformed organisms and to achieve this purpose it is necessary to know the susceptibility of the organism of interest to the selection agent (Cruz-Martín et al., 2001).

The selection agent in this study was the broad spectrum antibiotic hygromycin B because the binary vector inserted in *A. tumefaciens* contains the gene hph, a kinase that inactivates the antibiotic through a phosphorylation process (Cepeda and

Giraldo, 2011). Fungus that belongs to the genus *Fusarium* may present some differences in sensitivity to antibiotics; in fact, the MCI obtained in this study for *F. oxysporum* was 150 µg/mL of hygromycin while that for *F. solani* has been reported at 40 µg/ mL (De Oliveira et al., 2012).

4.4 Agrobacterium-mediated transformation of *Fusarium oxysporum*

Agrobaterium tumefaciens-mediated transformation has been used since the late 20th century to integrate a variety of genes into plants, and its use was later extended to the transformation of fungi, especially in the genus *Fusarium* (Mullins et al., 2001). Similarly, the expression of fluorescence proteins has been used to study host-pathogen interaction in several studies (Mullins et al., 2001; Caasi et al., 2010). In this research, an *F. oxysporum* isolate transformed to express the tdTomato fluorescence protein through the method of *Agrobacterium tumefaciens* mediated transformation (ATMT) and it was obtained from mycelium fragments with a transformation frequency of 1 transformed per 10^6 CFU. Mullins et al. (2001) proposed a transformation method using binary vectors with antibiotic resistance genes and obtain a production of 300 to 500 hygromycin B resistant transformed for every 1 x 10^6 conidia of *F. oxysporum* when the cells of *A. tumefaciens* grew in the presence of acetosyringone. The efficiency of the transformation is correlated with the co-culture time of the fungus spores with *A. tumefaciens* (Flores et al., 2015).

The plasmid that was used has genes for resistance to hygromycin B and kanamycin with eukaryotic promoters, which is why it serves as a selection agent for the transformed ones when these antibiotics are used.

4.5 Colonization of the transformed fungus in avocado roots

After 30 days from inoculation, the absence of root colonization of *F. oxysporum* was mainly observed in the cultivar Criollo; while the presence of mycelium around the root and in the root cut was observed in the cultivar Fuerte. This result is related to the differences at the genetic level existing between the two cultivars because avocado has been subject to different domestication processes that have shaped its genetic structure and diversity (Cañas-Gutiérrez et al., 2015; Corona-Jácome et al., 2016).

The root colonization percentages were lower for the cultivar Criollo; besides, the Kruskal Wallis test allowed to confirm differences in the response of the two cultivars evaluated, thus inferring that cultivar Criollo (Mexican race) has a greater tolerance to the pathogen. In fact, this type of cultivar has been shown to be tolerant to soil pathogens (Sánchez-González et al., 2019). In addition, according to Rincón-Hernández et al. (2011), the cultivar Criollo is often used as rootstocks because it has genes for resistance to the attack of pathogens that affect the root system.

5. Conclusion

In this research, a current biotechnological tool in the agricultural field was used, allowing evaluating aspects of the plant-pathogen relationship through the response of the cultivars Criollo and Fuerte to the inoculation of the *F oxysporum* transformed. According to the results, it can be inferred that the cultivar Criollo showed a greater tolerance to *F. oxysporum* root colonization, which corroborates the recommendation of its use as a rootstock for commercial avocado varieties. In addition, according to our knowledge, this is the first report of *F. oxysporum* affecting avocado seedlings in nursery in Ecuador.

To take advantage of this methodology, further studies using more isolates and avocado cultivars are needed. This method can be applied in breeding programs to select tolerant accesions during the first stages of plant growth. In addition, this biotechnological tool can be tested in other vascular pathogens to better understand plant-pathogen interactions.

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