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Uniformity in shoot development in avocado grafts and its importance in establishing levels of indirect resistance to *Phytophthora cinnamomi*

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ABSTRACT

Background / Objective. The level of resistance to *Phytophthora cinnamomi* in avocado germplasm can be evaluated indirectly through inoculation of the pathogen by wounding the stem. The objective of this work was to compare the conventional graft development method and the etiolated graft method to determine levels of indirect resistance to *P. cinnamomi* through the stem wound inoculation technique.

Materials and methods. In the study, three isolates of *P. cinnamomi* and two avocado genotypes with different levels of resistance to the pathogen were used, Duke-7 (medium resistant) and Hass (susceptible). Clonal multiplication of the genotypes was carried out with buds grafted on rootstocks propagated by Antillean avocado seeds. Inoculation was performed on the shoot at a height of 8 cm and the growth of the lesions was measured for 24 days. With the data, the Area Under the Disease Progress Curve (AUDPC) and the Coefficients Variation (CV) are calculated. The information was analyzed with a completely randomized experimental design with a 2*2*3 factorial arrangement (Method*Genotype*Isolation).

Results. The analysis of variance for the AUDPC showed no differences between methods (p=0.1881); However, there were differences between genotypes, isolates and between the genotype*method and genotype*isolation interactions (p≤0.05). With the conventional method, the development of the outbreaks was late (141-159 days) and the size of

the lesions was highly variable (CV=38.9-64.4%), being able to discriminate partial degrees of aggressiveness between isolates, but not levels of resistance between genotypes. The etiolated and greened shoots in the nursery, on the contrary, presented rapid growth (101-107 days) and greater uniformity in the lesions generated by the pathogen (CV=11.1-24.2%).

Conclusion. The development of etiolated shoots in avocado grafts is proposed as a rapid alternative method that can guarantee greater uniformity in the development of lesions within the experimental units of a treatment, thus achieving greater reliability when evaluating and selecting preliminarily. avocado genotypes with indirect resistance attributes to *P. cinnamomi*.

Keywords: *Persea americana*, etiolation, virulence, genetic improvement.

Introduction

The characterization and evaluation of avocado (*Persea americana*) (Lauraceae) phytogenetic resources constitute a strategic research area that can address challenges in current and future production systems (Ben-Ya'acov and Zilbersteine, 1999). One of the main limitations of cultivation today is root rot caused by the oomycete *Phytophthora cinnamomi* (Peronosporaceae). This pathogen destroys fine roots, affecting water and nutrient uptake, which leads to the development of secondary symptoms in the foliage and, in the medium term, plant death (Mora-Aguilera *et al*., 2007). Management of this problem is limited, and an integrated approach is recommended (Drenth and Guest, 2004; Ramírez-Gil *et al*., 2017). The use of rootstocks with resistance attributes to *P. cinnamomi* is proposed as one of the main options, as when combined with other management practices appropriately, it can be a sustainable solution in the medium and long term (Engelbrecht and Van den Berg, 2013).

The level of resistance to *P. cinnamomi* in avocado germplasm can be indirectly evaluated through the stem wound inoculation technique. This screening method uses vegetative propagation of avocado germplasm through buds grafted onto rootstocks developed from sexual seeds. When the grafted shoot reaches adequate development, a small wound is made in the stem, into which a PDA (Potato Dextrose Agar) disc with pathogen mycelium is inserted. The lesion progress is then evaluated over a specific time period (Dolan and Coffey, 1986; Gabor and Coffey, 1991; Rodríguez

et al., 2017). This technique has been proven as a preliminary alternative method for rapid and convenient detection of potentially resistant avocado genotypes to *P. cinnamomi*, due to its easy reproducibility and favorable correlation with the direct root inoculation method (Dolan and Coffey, 1986; Gabor and Coffey, 1991; Rodríguez-Padrón *et al*., 2018). However, the use of this screening technique must ensure the greatest uniformity in vegetative development of grafts, especially in size, degree of lignification, and stem diameters suitable for inoculation. This is to obtain less variation in lesion areas among experimental units of a genotype when inoculated with *P. cinnamomi* (Gabor and Coffey, 1991; Rodríguez *et al*., 2017).

The aim of this study was to compare and characterize two methods of shoot development in avocado grafts to determine levels of indirect resistance to *P. cinnamomi* using the stem wound inoculation technique. This was done to establish an appropriate method for multiplication and shoot development that allows for greater reliability when preliminarily evaluating and selecting avocado genotypes with attributes of resistance to the pathogen.

Materials and Methods

Study area. The study was conducted at the Palmira Research Center of AGROSAVIA, Valle del Cauca, Colombia (03°30′43.6″ N and 76°18′53.5″ W; 1001 m above sea level), from October 2019 to March 2020.

Seed conditioning and rootstock production. For rootstock production, seeds from West Indian avocado (*P. americana* var. *americana*) were used, sourced from recognized seed donor trees at the production center in Alvarado (Tolima, Colombia) (Berdugo-Cely *et al*., 2023; López-Galé *et al*., 2022). Seeds were manually extracted from healthy fruits at physiological maturity. To ensure cleanliness and disinfestation, the seed coat was completely removed. All seeds underwent a 1 cm apical cut to facilitate epicotyl emergence. Subsequently, they were disinfested with a solution of 2 cm^3 L^{-1} Fosetyl + Propamocard and 3 g L^{-1} Carboxin + Captan for 2 minutes.

The substrate used for germination was fine sand, previously solarized and disinfested with 2 cm^3 L^{-1} Fosetyl + Propamocard. After planting, the seeds remained under plastic cover and black shade cloth with 50% light reduction until the epicotyl emerged more than 1 cm, which indicated the optimal time for transplanting to nursery bags with substrate. The substrate

used for transplanting consisted of peat, rice husk, sand, and vermiculite in ratios of 4:2:1:1, respectively, previously solarized and disinfested with 2 cm^3 L^{-1} Fosetyl + Propamocard.

Vegetative propagation of avocado germplasm. Two avocado genotypes with different degrees of resistance and susceptibility to *P. cinnamomi* were selected from the Colombian Avocado Germplasm Collection (AGROSAVIA, Palmira Research Center). The selected genotypes were avocado var. 'Hass', identified as a susceptible genotype (Rodríguez *et al*., 2017; Sánchez-Gonzalez *et al*., 2019), and the standard clone 'Duke-7', reported as a moderately resistant genotype to *P. cinnamomi* (Barrientos-Priego *et al*., 2007; Kellam and Coffey, 1985). Clonal multiplication of the avocado genotypes was performed using terminal cleft grafted buds on previously seed-propagated rootstocks. The buds of each genotype were collected, processed, and disinfested following the recommendations of Rodríguez *et al*. (2017).

Conventional graft development method. The method followed guidelines established by Bernal-Estrada and Díaz-Díez (2020) for nursery propagation of planting material. Germinated West Indian avocado seeds were transplanted into black 6 L nursery bags filled with substrate. Hass and Duke-7 avocado buds were grafted at 20 cm above the rootstock stem base, ensuring a minimum rootstock diameter of ≥ 6 mm. Grafted seedlings were kept under plastic cover and black shade cloth (50% light reduction) until successful graft union and shoot growth reached > 30 cm in height with stem diameters ≥ 6 mm when measured 8 cm above the graft point (Figure 1A).

Etiolated graft development method. The method was adapted from the protocol described by Frolich and Platt (1972) for clonal rootstock propagation and the recommendations provided by Gabor and Coffey (1985) for evaluating avocado germplasm against *P. cinnamomi*. In this method, germinated seeds were transplanted into black nursery bags with a volumetric capacity of 0.5 L of substrate. Hass and Duke-7 avocado buds were grafted at a height of 3 cm from the rootstock stem base, ensuring a minimum rootstock diameter of ≥ 6 mm at this height. Grafted plants were transferred to a dark or etiolation chamber once graft success was observed and apical shoot development exceeded 1 cm, to ensure their viability before entering the etiolation chamber (Figure 1B).

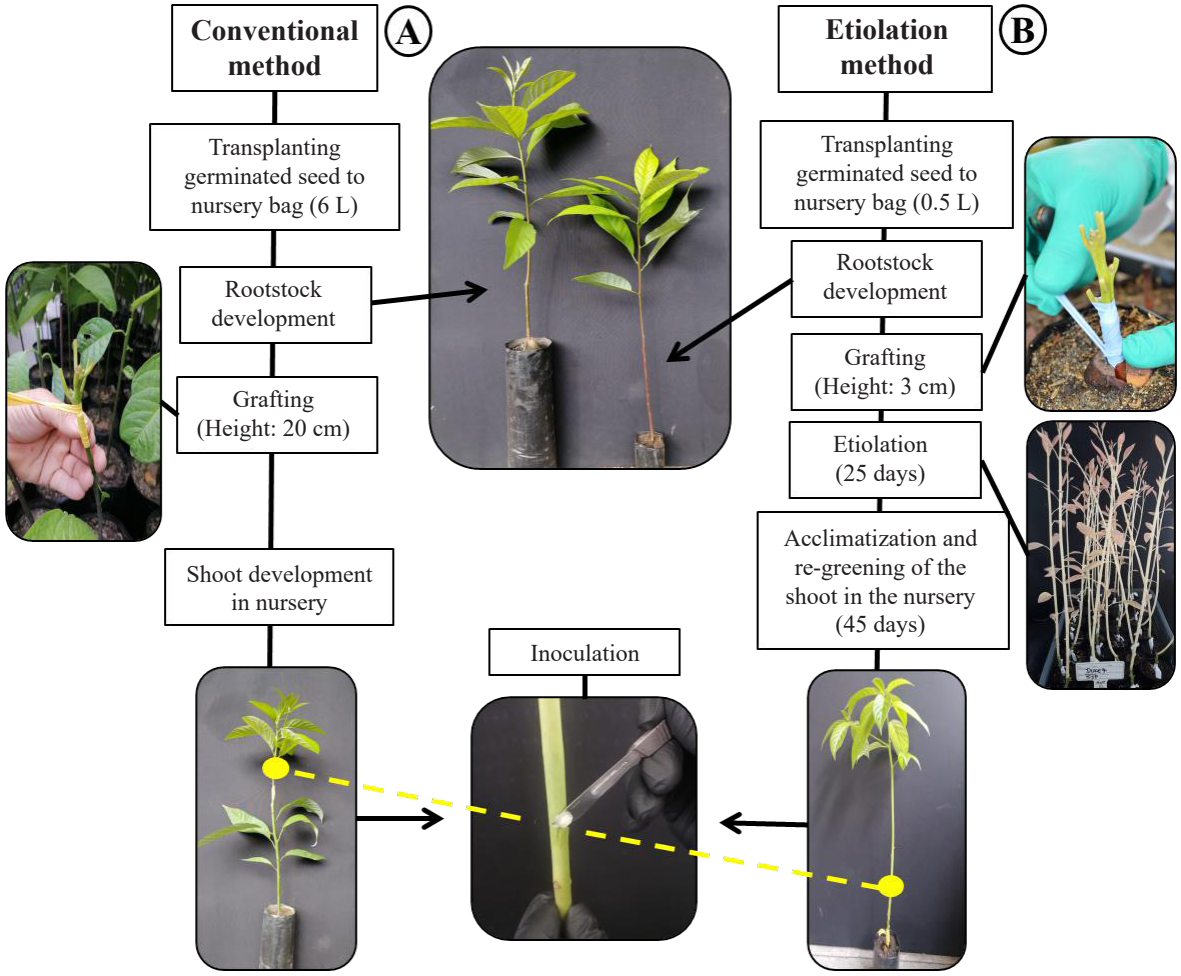

Figure 1. Propagation and shoot development process in avocado grafts. A) Conventional graft development method, B) etiolated graft development method. Yellow dots and lines indicate the location where the wound was made and *P. cinnamomi* was inoculated.

Shoot etiolation lasted approximately 25 days, during which the etiolation chamber maintained an average temperature of 25.8 °C (Range: 23.9–26.9 °C) and average relative humidity of 88.1% (Range: 76.1–93.5%). Etiolated seedlings were transferred to shade house conditions with plastic cover and black shade cloth (50% light reduction) when they reached heights greater than 30 cm and stem diameters ≥ 6 mm at 8 cm above the grafting point. The acclimatization and re-greening period for etiolated shoots was 45 days (Schmidt, 1986) (Figure 1B).

Isolates of *Phytophthora cinnamomi.* To obtain and produce *P. cinnamomi* inoculum, three isolates preserved in the working collection of

AGROSAVIA's phytopathology laboratory at the Palmira Research Center were reactivated. These isolates had been previously evaluated and classified as highly virulent in pathogenicity tests (Palacios-Joya *et al*., 2023; Rodríguez *et al*., 2017; Rodríguez-Polanco *et al*., 2015). The selected isolates were: Ag-A-041 collected in Tribunas Córcega district in the Risaralda department, Ag-A-003 from Rionegro municipality in the Antioquia department, and Tamb-009 collected in El Tambo municipality, Cauca department. All three *P. cinnamomi* isolates were obtained from root samples collected from avocado trees showing wilt symptoms.

Isolates were reactivated on green apples using Erwin and Ribeiro's (1996) method. In a laminar flow chamber, apples underwent surface disinfection with 96% alcohol and 1% sodium hypochlorite, followed by triple rinsing with sterile distilled water. A 1 cm deep hole was created in the apple's center using a sterilized cork borer. This cavity was filled with a PDA disk containing mycelium from each preserved isolate, then sealed with the extracted pulp and Parafilm tape. Apples were incubated in a humid chamber at 28°C until bark necrosis developed.

Small fragments (5 mm^2) of healthy and necrotic tissue from the apples were plated on Petri dishes containing *Phytophthora*-selective medium (PDA + fungicides + antibiotics) (Tsao and Guy, 1983). The three isolates were cultured at 28°C, and taxonomic identification was confirmed through macroscopic morphological characterization of colony growth and microscopic examination of vegetative and reproductive structures (Abad *et al*., 2023).

Inoculation. *P. cinnamomi* inoculation employed the stem wound technique (Dolan and Coffey, 1986; Gabor and Coffey, 1991; Rodríguez *et al*., 2017; Rodríguez-Polanco *et al*., 2015). A bark fragment measuring 10 mm long, 5 mm wide, and 1 mm deep was removed 8 cm above the grafting point. The wound received a 6 mm diameter PDA disk containing mycelium from one of three *P. cinnamomi* isolates, tested individually (Figure 1). Control plants received PDA disks without inoculum. Parafilm tape sealed the wound, securing the inoculum, preventing contamination, and facilitating pathogen colonization.

Data comparison and analysis parameters. Each method was evaluated for the time grafts took to reach the inoculation diameter (≥ 6 mm) 8 cm above the grafting point.

P. cinnamomi lesion areas were measured every three days for 24 days, recording length and width. The Area Under the Disease Progress Curve (AUDPC) and Coefficients of Variation (CV) were calculated from these

data. Disease symptoms, including necrosis, chlorosis, wilting, and graft death, were documented throughout the evaluation period. A WatchDog 2475 Mini Station monitored temperature (°C) and relative humidity (%) in the mesh house.

Histological sections of Hass and Duke-7 avocado graft stems were prepared to examine shoot tissue structure for each method prior to *P. cinnamomi* inoculation, following Andrade-Hoyos *et al*.'s (2015) methodology.

The study used a completely randomized design with a 2*2*3 factorial arrangement: shoot development methods, avocado genotypes, and *P. cinnamomi* isolates. Each treatment had three replicates, with three plants per replicate as the experimental unit. Analysis of variance (ANOVA) was conducted, with mean comparisons determined by Duncan's test ($p \leq 0.05$). Data analysis employed SAS version 9.4 for Windows.

Results and Discussion

Highly significant statistical differences were found in shoot development times between the evaluated methods ($F=12.18$; $p \leq 0.05$). In both avocado genotypes (Hass and Duke-7), shoots subjected to etiolation and subsequent re-greening under nursery conditions showed faster growth and development compared to the conventional method.

The minimum estimated diameter (6 mm) at 8 cm above the grafting point was achieved in etiolated Hass avocado shoots after 101 days on average, and in Duke-7 after 107 days. In contrast, shoots propagated by the conventional method reached the minimum diameter in Hass avocado after 141 days and in Duke-7 after 159 days on average (Table 1). This indicates that for Hass and Duke-7 avocados, obtaining appropriate stem diameters for *P. cinnamomi* inoculation through the etiolation method can reduce shoot development time by an average of 30.6% compared to shoots developed by the conventional method. Furthermore, graft etiolation ensured greater uniformity in shoot development, as evidenced by the low CVs obtained for Hass avocado (5.14%) and Duke-7 (5.51%) (Table 1).

Suitable diameters for inoculation (≥ 6 mm) in the etiolation chamber were obtained between 19 and 25 days after grafting in both genotypes. Additionally, it was confirmed that 45 days of acclimatization and re-greening of etiolated tissues were adequate to ensure lignification and bark thickness in Hass and Duke-7 avocado shoots (Schmidt, 1986) (Figure 2), attributes required for artificial inoculations of avocado stems with *P. cinnamomi* (Rodríguez *et al*., 2017).

Table 1. Estimated vegetative development (days) of Hass and Duke-7 avocado grafts propagated by the conventional method and etiolation method.

Method	Genotype	Vegetative development (days)	
		Average ± S.D.[x]	CV[y] (%)
Conventional	Hass	141 ± 12.8 a*	9.07
	Duke-7	159 ± 14.1 a	8.86
Etiolation	Hass	101 ± 5.2 b	5.14
	Duke-7	107 ± 5.9 b	5.51
		LSD[z] = 38.2	

*Mean values with different letters are statistically different.
[x]S.D.: standard deviation
[y]CV: coefficient of variation
[z]LSD: least significant difference

Tissue etiolation is a natural growth response in avocado plants that occurs in the absence of light. This state induces structural changes at both morphological and anatomical levels, promoting cellular development (Hiti-Bandaralage *et al*., 2017). Etiolated shoots of Hass and Duke-7 genotypes exhibited structural increases in stem diameters, particularly in cortical width and xylem rays, facilitated by cell enlargement (Figure 2). These results partly explain the rapid shoot development observed in both genotypes within the etiolation chamber and align closely with Schmidt's (1986) observations in etiolated stems of the tree species *Tilia tomentosa* (Malvaceae).

Both shoot development methods and avocado genotypes showed similar responses to the three *P. cinnamomi* isolates. External bark rot manifested as dark brown spots or cankers with whitish exudates, while internal bark exhibited advancing lesions and brown or reddish tissue discoloration. These findings corroborate reports by Fischer *et al*. (2020) and Rodríguez-Padrón *et al*. (2018), confirming that *P. cinnamomi* mycelium inoculation via PDA disks induces bark and vascular tissue death comparable to zoospore inoculation (Van der Merwe *et al*., 1990).

P. cinnamomi was consistently re-isolated from all inoculated plants, confirming its role in avocado shoot necrosis progression. On PDA, the isolates displayed similar petaloid colony growth patterns with white, creeping mycelium (Figure 3A). Microscopic examination revealed coenocytic, branched hyphae with globose and subglobose swellings (Figure 3B), terminal thick-walled globose chlamydospores (Figure 3C), and unbranched sporangiophores bearing non-papillate, ellipsoid to lemon-

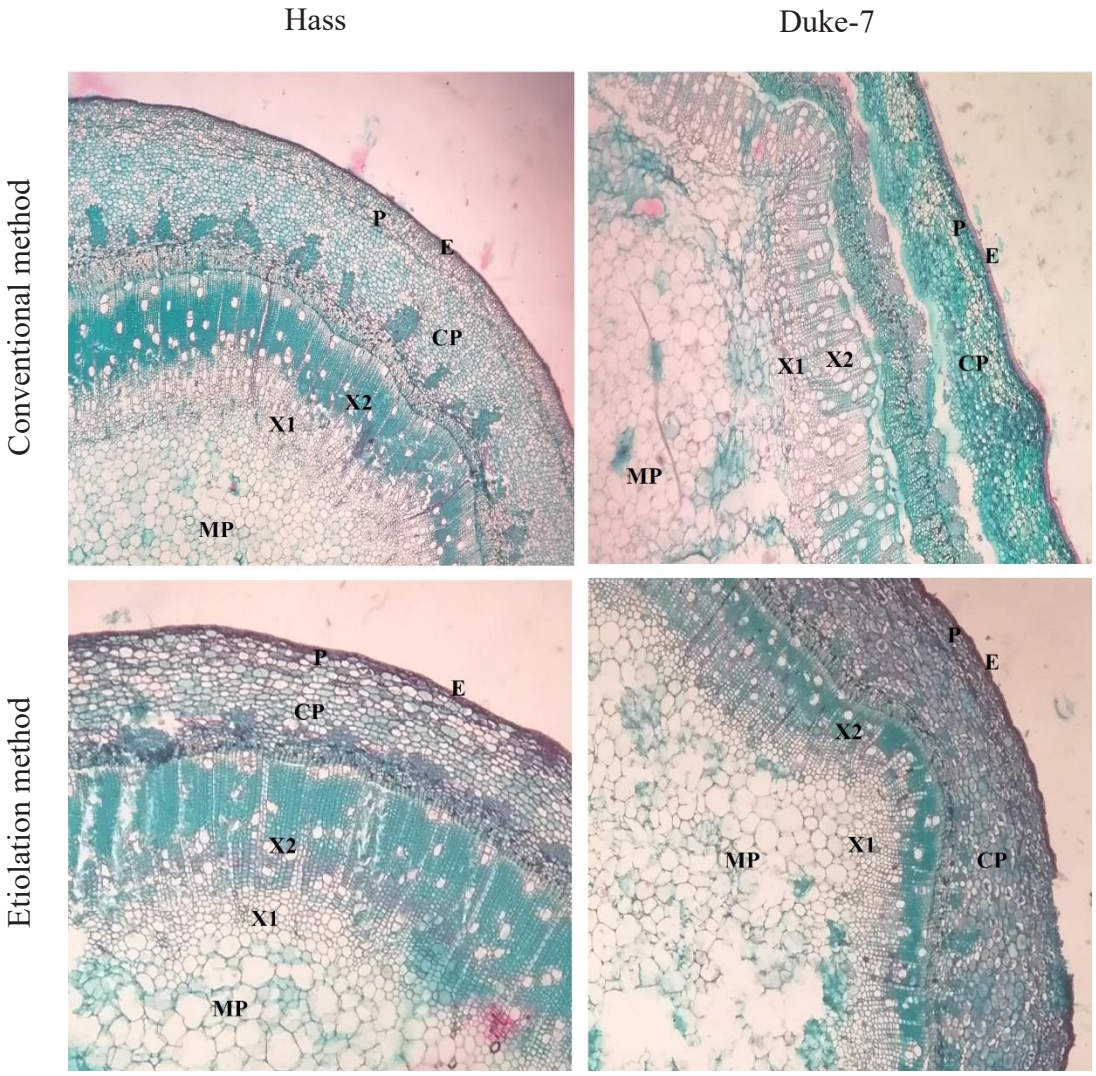

Figure 2. Micrographs of histological sections of Hass and Duke-7 avocado stems developed by the conventional method and the etiolation method (40X). (MP) Medullary parenchyma, (X1) Primary xylem, (X2) Secondary xylem, (CP) Cortical parenchyma, (P) Periderm, (E) Epidermis.

shaped sporangia (Figure 3D). Sporangia measured 48.2-57.3 µm in height and 32.7-33.4 µm in width, aligning with Abad *et al*.'s (2023) descriptions of *P. cinnamomi*.

Chlorosis, wilting, and decline in leaves and stems appeared 9 days post-inoculation (dpi) across both shoot development methods. Shoot death emerged at 15 dpi in conventionally propagated seedlings but delayed until 22 dpi in etiolated shoots. Hass exhibited the highest shoot mortality,

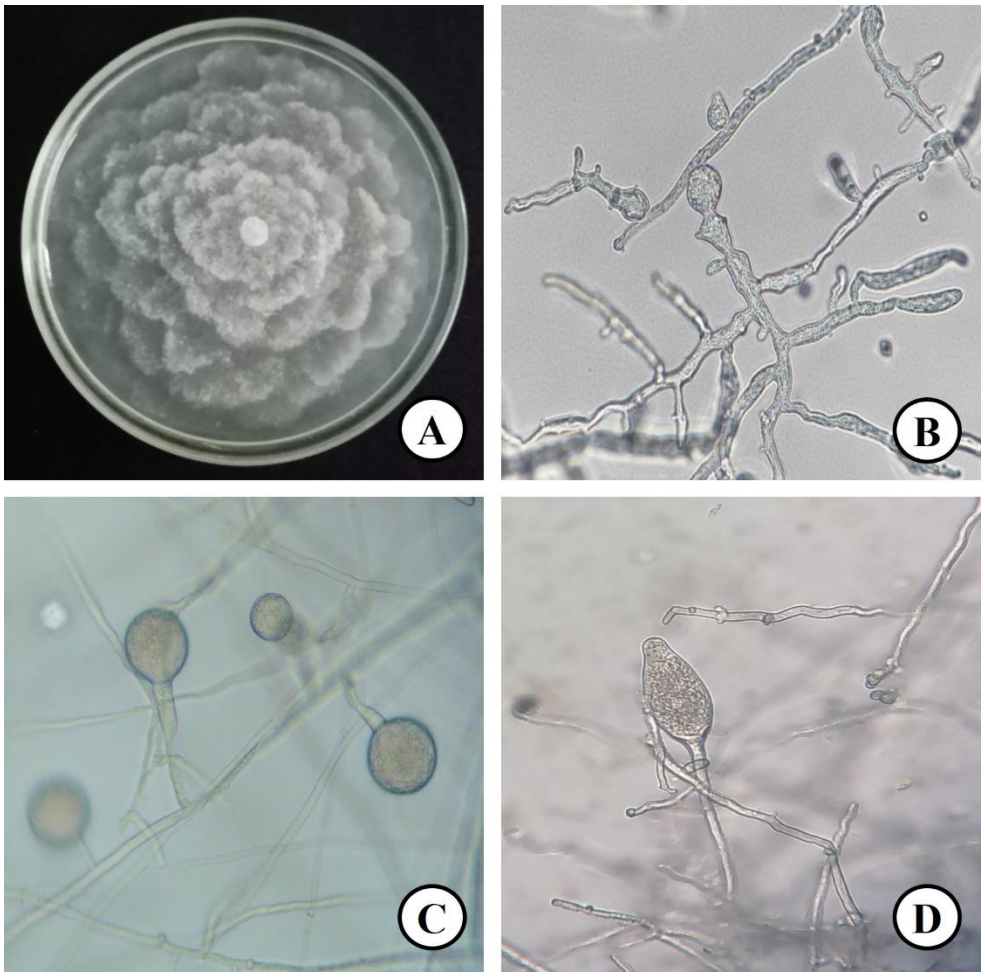

Figure 3. Macroscopic and microscopic characteristics of *P. cinnamomi* isolate A-Ag-041. (A) Colony growth on PDA culture medium, (B) mycelium (hyphae), (C) chlamydospores, (D) sporangium.

particularly in conventionally propagated shoots (Table 2). Mortality coincided with lesion progression, occurring once damage fully encircled the stem (Fischer *et al*., 2020). Control plants remained free of necrosis or lesion progression beyond the cut point.

The mesh house climate facilitated disease symptom development. Average temperature was 23.3°C (17.2–31.2°C range) with 71.6% relative humidity (42.7–97.8% range), consistent with conditions reported by Andrade-Hoyos *et al*. (2012), Fischer *et al*. (2020), and Rodríguez *et al*. (2017) in *P. cinnamomi* infection studies.

Table 2. Mortality of shoots in Hass and Duke-7 avocado grafts propagated by the conventional method and etiolation method, and inoculated with *P. cinnamomi*.

Method	Hass		Duke-7	
	Number of plants inoculated	Mortality	Number of plants inoculated	Mortality
Conventional	27	5 (18 %)	27	2 (7 %)
Etiolation	27	3 (11 %)	27	0 (0 %)
Total	54	8 (14.8 %)	54	2 (3.7 %)

The analysis of variance for the mean AUDPC values showed no significant differences between methods (F=1.84; p=0.1881). However, differences were observed between genotypes (F=13.52; p=0.001), isolates (F=3.47; p=0.047), and in the interactions of genotype*method (F=4.22; p=0.05), and genotype*isolate (F=3.34; p=0.037). This necessitated independent analysis of each treatment for each method and avocado genotype.

In shoots developed by the conventional method, overlapping was observed in the average lesion growth curves between isolates over time (Figure 4A and B). The lesion size (24 dpi) for Hass avocado in the three isolates ranged between 1.78 and 2.40 cm, while in Duke-7 it varied between 1.70 and 1.89 cm. In these shoots, no significant differences were found between genotypes according to AUDPC (F=0.73; p=0.4091). In Hass avocado shoots, the Tamb-009 isolate was the most aggressive, while isolates Ag-A-041 and Ag-A-003 had lower and statistically similar AUDPC. In Duke-7, however, no differences in aggressiveness between isolates could be discerned (Figure 5A). In shoots propagated by the conventional method, leaves and lateral branches were frequently found in the inoculation zone, complicating evaluation (Figure 6A) and possibly impeding the progression of necrotic lesions generated by the oomycete.

In shoots developed by the etiolation method, the lesion size (24 dpi) in Hass avocado for the three isolates ranged between 1.68 and 2.35 cm, while in Duke-7 it varied between 1.02 and 1.45 cm. In this method, highly significant statistical differences were found between genotypes (F=4.22; p<0.05) and between *P. cinnamomi* isolates according to mean AUDPC values (p≤0.05). Lesion development for the three isolates was greater in the Hass genotype compared to Duke-7. In both genotypes, lesion size exhibited exponential growth during the first 12 dpi, and from 15 dpi onward, a relatively stable growth pattern was observed until the end of the study (Figure 4C and D). Duncan's mean comparison test for both genotypes identified the Tamb-009 isolate as the most aggressive, followed by isolates Ag-A-041 and Ag-A-003 as the least aggressive (Figure 5B).

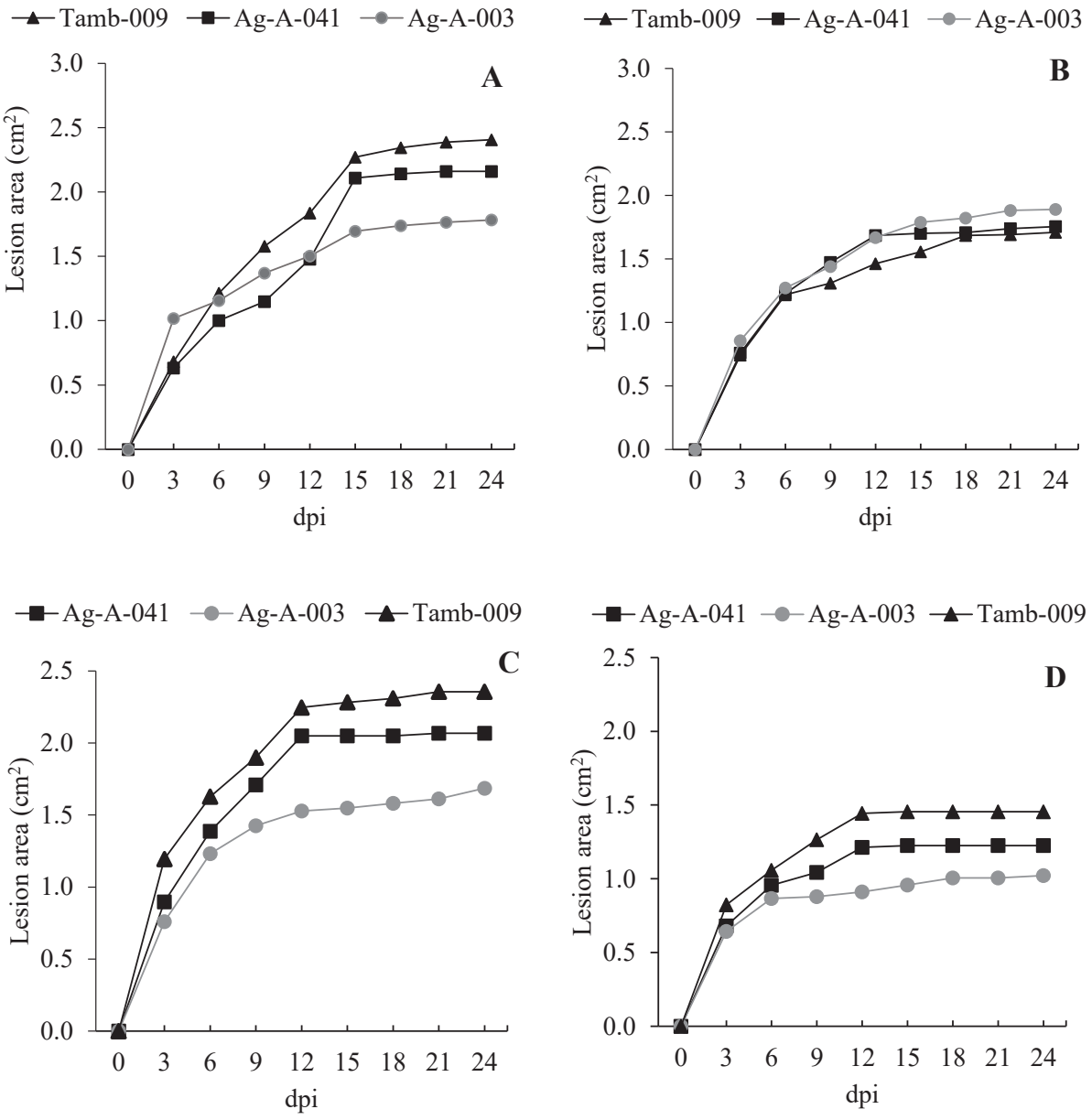

Figure 4. Progression of lesion area in Hass and Duke-7 avocado grafts developed by the conventional method and etiolation method and inoculated with three *P. cinnamomi* isolates. (A) Conventional Hass genotype, (B) conventional Duke-7 genotype, (C) etiolated Hass genotype, (D) etiolated Duke-7 genotype.

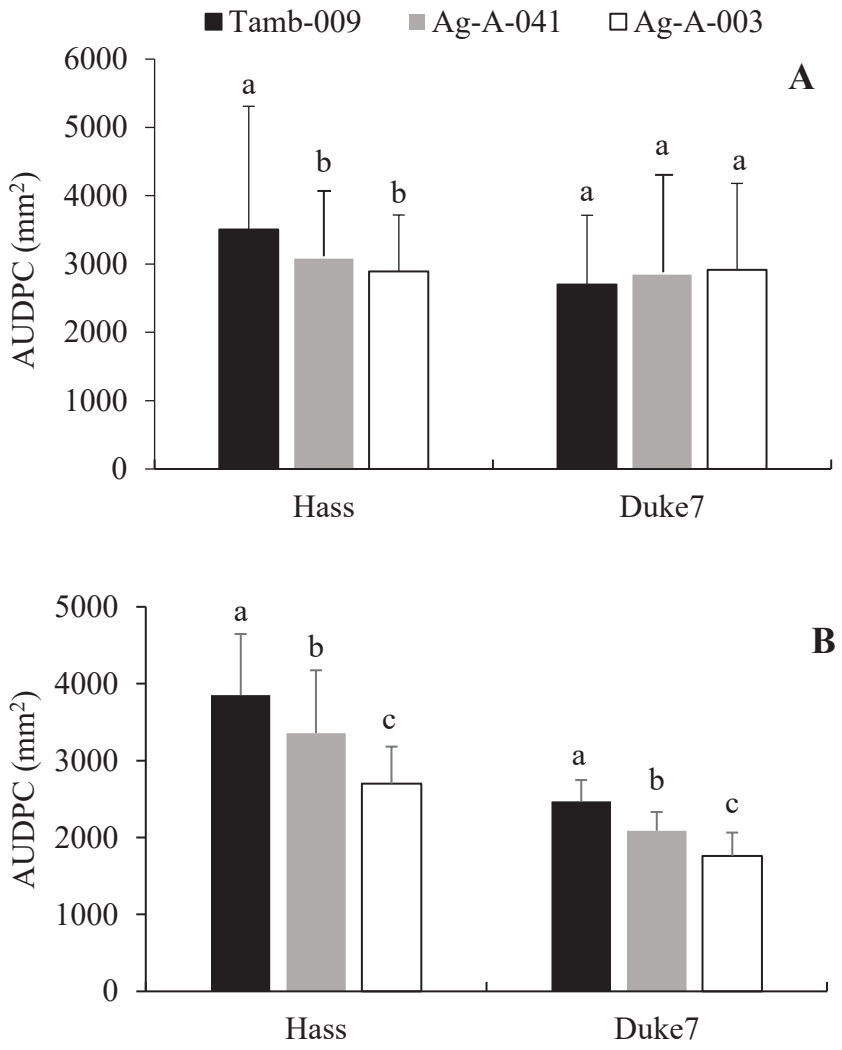

Figure 5. Disease Progress Curve Area Under the Curve (AUDPC) in Hass and Duke-7 avocado grafts developed by the conventional method (A) and the etiolation method (B).

Regardless of the shoot development method evaluated, the 'Hass' genotype showed the largest lesion development, AUDPC (3231.7 mm²), and graft death (14.8%), confirming its high susceptibility to *P. cinnamomi* (Rodríguez *et al*., 2017; Sánchez-Gonzalez *et al*., 2019). Similarly, the Duke-7 genotype demonstrated greater resistance to *P. cinnamomi*, with smaller lesion size, AUDPC (2463.6 mm²), and graft death (3.7%) (Barrientos-Priego *et al*., 2007; Kellam and Coffey, 1985). These differences in resistance and susceptibility to *P. cinnamomi* between the Duke-7 and Hass genotypes

Figure 6. Development of necrosis in avocado grafts inoculated with *P. cinnamomi*. (A) Variation in necrosis area in Hass avocado grafts developed by the conventional method (12 dpi). (B) Uninoculated control in Hass avocado graft developed by the conventional method. (C) Variation in necrosis area in Hass avocado grafts developed by the etiolation method (12 dpi). (D) Uninoculated control in Hass avocado graft developed by the etiolation method. Red arrows indicate the presence of vegetative structures that hinder proper evaluation of the necrosis area.

were particularly pronounced in grafts developed through the etiolation method.

Moreover, the high infectivity of the Tamb-009 isolate was confirmed in the evaluated trials, even surpassing the Ag-A-041 isolate, which has previously been verified as highly pathogenic and aggressive in avocado stem inoculation studies. For instance, Rodríguez-Polanco *et al*. (2015), using stem wound technique on Hass avocado grafts, evaluated 22 isolates of *P. cinnamomi* from various avocado-producing regions in Colombia, finding that the Ag-A-041 isolate was the most aggressive (AUDPC=64104 mm^2), achieving stem necrosis lengths between 15 and 20 cm. Similar results were reported by Rodríguez *et al*. (2017), who, while evaluating 21 avocado

genotypes with three isolates of *P. cinnamomi* using the stem wound technique, found that the Ag-A-041 isolate had the highest aggressiveness and infection rate (AUDPC=4086 mm²).

The uniformity in lesion size caused by the three isolates of *P. cinnamomi* in the two avocado genotypes was greater in shoots developed through the etiolation method (Figure 6). These results were supported by lower CV percentages of AUDPC for etiolated shoots of the Hass and Duke-7 genotypes, with variation levels ranging between 11.1% and 24.2%; whereas, in shoots developed through the conventional method, the variation percentages ranged between 38.9% and 64.4% for both genotypes (Table 3). This confirms greater uniformity in lesion size in etiolated shoots when experimental units of a genotype are subjected to a specific treatment (e.g., isolation), resulting from lower variation in the vegetative development of grafts and possibly enhanced by the enlargement and better cellular arrangement at the histological level in etiolated shoots, especially in the cortical parenchyma (CP) (Figure 1), where the PDA disk with *P. cinnamomi* mycelium is cut and inserted.

Table 3. Mean values and Coefficient of Variation (CV) of the Area Under the Disease Progress Curve (AUDPC) for three *P. cinnamomi* isolates inoculated in Hass and Duke-7 avocado grafts developed by the conventional method and the etiolation method.

Method	Genotype	Isolation	AUDPC	CV (%)
Conventional	Hass	Tamb-009	3504.4	60.0
		Ag-A-041	3081.6	64.4
		Ag-A-003	2890.9	38.9
	Duke-7	Tamb-009	2698.1	37.6
		Ag-A-041	2846.4	51.4
		Ag-A-003	2915.6	43.3
Etiolation	Hass	Tamb-009	3853.8	20.0
		Ag-A-041	3358.7	24.2
		Ag-A-003	2700.8	17.8
	Duke-7	Tamb-009	2471.4	11.1
		Ag-A-041	2089.5	11.6
		Ag-A-003	1760.5	17.2

Conclusions

Avocado plants produced through grafting and subsequent shoot etiolation demonstrated rapid growth (101-107 days), greater stem diameter uniformity (6 mm, CV=5.14-5.51%), and more consistent lesion development

within experimental units for each genotype when inoculated with *P. cinnamomi* (CV=11.1-24.2%). This enhanced consistency yields more reliable results when evaluating resistance-susceptibility in avocado germplasm. The etiolated shoot method also effectively differentiated resistance levels between genotypes and aggressiveness among *P. cinnamomi* isolates.

In contrast, the conventional graft development method resulted in higher variability in lesion growth (CV=38.9-64.4%) in response to *P. cinnamomi* infection. This method partially distinguished degrees of isolate aggressiveness but failed to discriminate resistance levels between genotypes.

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