Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

Desarrollo y validación de escalas de severidad del complejo marchitez del aguacate causado por *Phytophthora cinnamomi*, *Verticillium dahliae* y el desorden hipoxia-anoxia y sus respuestas fisiológicas en plantas de aguacate

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ABSTRACT

Avocado wilt complex (AWC) is the most important disease in this crop. AWC may be caused by different causal agents that induce similar symptoms. Accurate scales of disease development (SDD) and physiological changes may be of special importance for the diagnosis and management of AWC. The objective of this work was to design and calibrate a specific SDD for the most common causal agents associated with AWC in Colombia, *Phytophthora cinnamomi* Rands and *Verticillium dahliae* Klebahn, and the hypoxia-anoxia disorder in both seedlings in net house and adult plants under field conditions. Furthermore, physiological responses to infection were determined. The disease was monitored under field and net house conditions. Shoot symptoms were recorded and quantification of inoculum in infected soil and tissue was performed. The visual scale was described based on external symptoms and calibrated with the inocula values by regression analysis. In the net house, net photosynthesis, stomatal conductance, and transpiration were measured during the different stages of disease development. The three causal agents induced a progressive reduction of net photosynthesis, stomatal conductance, and transpiration. The designed scales can be a valuable tool for epidemiological use and support in the diagnosis and management of AWC.

Key words: epidemiological analysis, disease measurement, severity calibration, inoculum, disease management.

RESUMEN

El complejo marchitez del aguacate (CMA) es la enfermedad más importante de este cultivo. El CMA puede ser causado por distintos agentes causales que inducen síntomas similares. Las escalas de desarrollo de la enfermedad (EDE) y los cambios fisiológicos pueden ser de gran importancia para el diagnóstico y manejo del CMA. El objetivo de este trabajo fue diseñar y calibrar EDE específicas para los agentes causales más comunes asociados al CMA en Colombia, *Phytophthora cinnamomi* Rands y *Verticillium dahliae* Klebahn, y el desorden hipoxia-anoxia en plántulas de invernadero y plantas adultas en condiciones de campo. Además, se determinó la respuesta fisiológica durante la infección. La enfermedad se monitoreó en condiciones de campo e invernadero. Los síntomas de la parte aérea de la planta fueron registrados y se cuantificó el inóculo en el suelo y tejido afectado. Se describieron las escalas visuales basadas en síntomas externos las cuales fueron calibradas con la cantidad de inóculo mediante análisis de regresión. En invernadero, se midieron la fotosíntesis neta, la conductancia estomática y la transpiración durante diferentes etapas del desarrollo de la enfermedad. Los tres agentes causales de enfermedad indujeron una reducción progresiva de la fotosíntesis neta, la conductancia estomática y la transpiración. Las escalas diseñadas pueden ser una herramienta valiosa para su uso epidemiológico y de apoyo en el diagnóstico y manejo del AWC.

Palabras clave: análisis epidemiológico, medición de enfermedad, calibración de la severidad, inóculo, manejo de la enfermedad.

Introduction

Avocado wilt complex (AWC) is a disease of high economic importance. It has been called a complex because the expression of the characteristic symptoms in the shoot can be easily confused, since they can be induced by different pathogens and abiotic disorders. Therefore, the causal agents and disorders involved are difficult to identify under traditional diagnostic practices, especially in the first stage of development of the disease. This may lead to erroneous

management practices that worsen the problem and cause large economic losses (Ramírez-Gil *et al*., 2017; Rodríguez *et al*., 2017; Hardham and Blackman, 2018; Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2019). AWC can affect various tissues in the avocado, especially the roots, inducing several symptoms. A specific description of symptoms with different causal agents and disorders associated with AWC are presented by Ramírez-Gil (2018), and Ramírez-Gil and Morales-Osorio (2019).

Despite the importance of the disease, not all causal agents or disorders associated with AWC have the same relevance. This may be due to their pathological fitness components or epidemiology within the productive system. The microorganism *Phytophthora cinnamomi* Rands is reported as the most important causal agent of the AWC at a world-wide level (Zentmyer, 1984; Ramírez-Gil *et al*., 2017; Hardham and Blackman, 2018). The fungus *Verticillium* sp. has been less studied, but has been increasingly recognized as important because of problems of root rot associated with the disorder hypoxia-anoxia, especially under tropical conditions in Colombia (Ramírez-Gil *et al*., 2017; Rodríguez *et al*., 2017; Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2019). Hypoxia-anoxia is not only considered a disorder that causes the death of avocado plants, but it can also be a stress factor that is highly related to the presence of *P. cinnamomic* (Stolzy *et al*., 1967; Ploetz and Schaffer, 1989; Sanclemente *et al*., 2014; Ramírez-Gil and Morales-Osorio, 2018). This disorder is associated with soil moisture, which reduces the gas content in the porous space of the soil volume (Stolzy *et al*., 1967; Sanclemente *et al*., 2014; Ramírez-Gil and Morales-Osorio, 2019).

To accurately score and evaluate a disease, the availability of an appropriate scale of severity is important. In the case of AWC, the information about scales for measuring disease development is limited mostly to the AWC caused by *P. cinnamomi*, such as the ones reported by Darvas *et al*. (1984) and Coffey (1991) for adult plants. Appropriate scales are not known to evaluate the disease when it is caused by *Verticillium* sp. or the disorder associated with hypoxia-anoxia. Another limitation of these described scales is that they only report the expression of symptoms in the shoot and not the effects caused at the root level. In addition, they are not calibrated for use under different environmental conditions and amount of inoculum. Pathogens and disorders associated with the root and stem, including those of biotic and abiotic origin in the avocado, alter the host plant's metabolism at the morphological and physiological levels. Avocados grown under conditions of hypoxia-anoxia in the soil or with a combination of *P. cinnamomi* infections and

hypoxia-anoxia may show symptoms of stunted growth, decreased stomatal conductance, lower net CO_2 assimilation, reduced photosynthetic rates, accelerated senescence, and other physiological responses (Ploetz and Schaffer, 1989; Reeksting *et al*., 2014; Sanclemente *et al*., 2014).

Based on these problems, this research had two objectives: (1) to develop and validate scales of disease development for the most common causal agents and disorder (*P. cinnamomi*, *Vertcillium dahliae*, and hypoxia-anoxia) associated with AWC under tropical conditions in Colombia under field and net house conditions. These include the dynamics through time of the amount of inoculum, the degree of tissue affected in the root and shoot of the plant. (2) To identify the plant physiological responses in seedlings affected by the three causal agents under semi-controlled conditions.

Materials and methods

Location

For the development and calibration of disease scales for the avocado wilt complex (AWC) that is caused by *P. cinnamomi*, *V. dahliae*, as well as for the hypoxia-anoxia disorder, two evaluations were performed. The first was carried out under field conditions. We selected three lots planted with avocado cv. Hass grafted on 6-year-old West Indian rootstock at a planting distance of 7x7 m. The second part of the work was performed in the laboratory of "Fitotecnia Tropical" and the net house at the Universidad Nacional de Colombia, Medellin campus. Details of edaphoclimatic conditions are shown in Supplementary material 1 and 2. The study under field conditions was carried out between 2012 and 2013 and corresponded to four periods of evaluation, performed during the rainy (March-April and September-October) and dry (November-January and June-August) seasons.

Development and validation of specific scales for AWC under field and net house conditions

To develop the scales for AWC under field conditions, diagnostic procedures reported by Ramírez-Gil and Morales-Osorio (2019) were followed, based on plant symptoms and microorganism isolation in semi-selective media. Isolation of *P. cinnamomi* was performed in aseptic vegetable juice (V8-180 ml L^{-1}) with the addition of agar (24 g L^{-1} Difco, USA), ampicillin (200 µg L^{-1}), chloramphenicol (20 µg L^{-1}), and benomyl (100 µg L^{-1}) (V8-AACB). Isolation of *Verticillium* sp. was performed in acidified potato dextrose agar plus lactic acid (PDA-A) (Difco, USA) and vegetable juice agar (V8-AE) (Difco, USA) supplemented with antibiotic

(streptomycin 100 µg L⁻¹). Morphological characterization at the genus and species level was performed using the keys of Barnett and Hunter (1972) and Seifert *et al.* (2011) for fungi, and Erwin and Ribeiro (1996) for *Phytophthora* spp. For *P. cinnamomi* and *Verticillium* sp., the identifications were confirmed by sequence analysis of the genomic ITS regions, using the primer pairs ITS5-ITS4 and ITS1-ITS4 and the procedures established by White *et al.* (1990). We selected *V. dahliae* because it was the most commonly identified in the evaluated plots. For hypoxia-anoxia, shoot symptoms, root dissection, and verification of microorganism′s presence or absence were determined (Ramírez-Gil and Morales-Osorio, 2019).

Plants infected with *P. cinnamomi* and *V. dahliae* under natural conditions were selected. The selection of plants affected with hypoxia-anoxia were based on two concepts: (1) plants planted in soil with low slope (less 10%) (Ramírez-Gil, 2018) and (2) plants with high accumulation of water in the soil profile (more than 70% of water saturation) using volumetric humidity values quantified in a V2 moisture sensor (analogy, DF Robot™, reference SKU:SEN0114) with serial communication to an Arduino UNO programming card, and collecting data with a serial communication terminal (Ramírez-Gil *et al.*, 2018). For each pathogen and disorder, a randomized complete block design was used with three blocks (each plot) and each block had three replicates, where the experimental unit consisted of five plants. All the infected plants were left under field conditions with no disease management procedure until they died of these pathologies. Care was taken to avoid the presence of another type of microorganism, based on the isolation of the selected plants and the reduction of the contact with the staff of the plantations.

Infected but asymptomatic plants were selected within the cultivated plots. For each measure five plants were selected with the same degrees of disease or disorder development so as to evaluate the root dynamics. These plants were evaluated once. A detailed description of the symptomatology developed through time in the shoots of the selected plants was registered, such as changes in leaf color, presence of withered leaves, defoliation, and affected tissues. This part was complemented with an evaluation of the root system. The number of affected roots was determined by destructive sampling introducing a stainless-steel cylinder (100 cm³ volume) at 10 points distributed around a diameter of 3 m equidistant from the base of the stem of the plant at a depth of 60 cm. The material removed was washed with tap water. The avocado roots were selected and a determination of their phytosanitary status as non-viable diseased roots (necrotic) and viable healthy roots (white) was carried out

(Ramírez-Gil and Morales-Osorio, 2019). The remaining root samples were kept for inoculum quantification (*P. cinnamomi* and *V. dahliae*) in the laboratory. For the pathology caused by *V. dahliae*, the affected shoot (stem and branches) was also considered and evaluated looking for necrosis or other symptoms.

A definition of the levels of the scale for disease development was carried out by photographic registration and symptom description. A scale from 0 to 5 was assigned, in which the first value (0) indicated an absence of the disease and (5) indicating a dead or irreversibly dying plant. Other values were assigned according to the progress of the symptoms in roots and shoots (Tabs. 1-3). From all of these data the corresponding specific shoot scales were determined (Figs. 1-3).

For the experiments in the net house, a completely randomized experimental design was used with five replicates per treatment. The experimental unit consisted of five seedlings and the evaluation had two repetitions through time. For the net house evaluation, strains of *P. cinnamomi* (Code: PCSOC1) and *Verticillium* sp. (*V. dahliae* Code: VAC3) were obtained from the collection of avocado pathogenic strains kept at the "Fitotecnia Tropical" Laboratory at the Universidad Nacional de Colombia, Medellin campus, which had been morphologically and molecularly characterized. For the reproduction of hypoxia-anoxia conditions, water was applied until 70-90% of water saturation (Ramírez-Gil and Morales-Osorio, 2019) in an Andisol soil (Supplementary material 1). Reproduction of the AWC was previously verified on specimens of avocado cv. Hass for the two causal agents and hypoxia-anoxia disorder.

Avocado seeds of undamaged cv. Hass of similar size were collected. They were incubated for germination in autoclaved quartz (0.1 MPa and 121ºC for two cycles of 1 h each) and maintained at 50-70% relative humidity. When seedlings had five fully expanded leaves and the secondary root system showed healthy roots (not necrotic) by visual inspection, cotyledons were removed to induce more root formation (Ramírez-Gil and Morales-Osorio, 2019). Seedlings were then transplanted to plastic pots with a 2 kg capacity on a wet basis. The potting soil was an Andisol from the municipality of El Peñol, Antioquia, Colombia, the description of which is presented in Supplementary material 1. The soil was autoclaved (0.1 MPa and 121ºC, for two cycles of 1 h each). Plants were kept under net house conditions, at 40-50% of moisture potential before carrying out the inoculation and reproduction of the hypoxia-anoxia conditions.

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

87

Inoculation was performed by adding an aqueous suspension of 200 ml (sterile distilled water) containing inoculum to a final concentration of 1×10^5 ml^{-1} infective propagules (conidia) of *V. dahliae*, and 1×10^3 ml^{-1} infective propagules (sporangia) of *P. cinnamomi*. The inocula were added to the root system of each seedling in four equidistant points in each pot (Ramírez-Gil and Morales-Osorio, 2019). Inoculum was grown in potato-dextrose agar medium (PDA-Difco, USA) at 22°C for 10 d, and added to sterile distilled water and dispersed by manual agitation. To achieve a condition of hypoxia-anoxia the soil used for seedling growth was kept at 70-90% of water saturation based on the process described before.

For the definition of the levels of disease development for these two pathologies and hypoxia disorder in net house seedlings, the procedure was similar to the one described above for adult plants in the field, in which symptomatology was monitored in the shoot and at the root level with the modification that the whole seedling was sampled for root analysis. The defined scale consisted of values from 0 to 3, where the value 0 corresponded to healthy plants and 3 to dead or irreversibly damaged to dead plants. With these data, the description of the scale (Tabs. 1-3) and its visual evaluation were defined (Figs. 1-3).

The inoculum and progress of affected tissues present in each of the stages of the disease development defined in Tables 1, 2, and 3 were quantified. For *P. cinnamomi,* 5 g of roots were randomly collected from the sample obtained with the cylinder and these were disinfected (Ramírez-Gil and Morales-Osorio, 2019). The roots were macerated in 200 ml of sterile distilled water. Serial dilutions (1 x 10^{-1} x 10^{-5} of sterile distilled water w:v) were prepared. One ml of each dilution was spread in Petri dishes with V8-AACB. For *V. dahliae,* 5 g of a mixture of roots and stem tissues was obtained and processed as described for *P. cinnamomi.* This was then seeded in Petri dishes with PDA-E. For these two microorganisms, the amount of inoculum expressed as colony forming units (CFU) was determined by direct counting from the dilutions performed. For the conditions of hypoxia-anoxia, the level of the disease was quantified as the percentage of dead roots with purple coloration in the inner part (Ramírez-Gil and Morales-Osorio, 2019). The plants with roots affected with a microorganism (i.e. *P. cinnamomi*) were discarded for this analysis.

The calibration for the disease development scales for these two pathogens and the disorder in adult plants under field conditions and seedlings under net house conditions was performed by regression analysis. The analysis was based on the relationship between the scale determined in the shoot and root and the amount of inoculum. The presence of the microorganism in affected tissues for which the scale was the dependent variable, and the amount of inoculum was the independent variable. The principles of normality of residuals (Kolmogorov-Smirnov test), homoscedasticity of residual variances (Levene test) and the uncorrelation of the residual (Durbin-Watson test) were evaluated. The model that best fits the data was chosen using the statistical parameters of correlation coefficient, coefficient of determination, *P* value ($P<0.05$) and the *Akaike information criterion* (AIC). In order to comply with the principles, data associated with the amount of inocula were logarithmically (ln) transformed. As a consequence, in healthy plants in which the inoculum was zero, this value was assumed to be 1 since the function (ln) does not present a real value for 0. In order not to alter the results, 1 was added to each scale. The analysis was run on the free software R (R Development Core Team, 2019).

As a complement to the previous analysis in each of the evaluated seasons (dry-rainy) the average time in days to move from one stage of the disease scale to another, the associated inocula, and the colonization on tissues were determined, according to the scale of disease development proposed. This evaluation was also performed for seedlings in the net house, but without the effect of the dry-rainy season. In the case of the disorder associated with the conditions of hypoxia-anoxia, it was only evaluated during the rainy season since its presence occurs only under conditions of high soil moisture (Ramírez-Gil and Morales-Osorio, 2019).

Simulation of root growth under field and net house conditions and AWC infections

A theoretical model was developed to simulate the effect of root infection by *P. cinnamomi* and *V. dahliae* and the hypoxia-anoxia disorder on root growth under field and net house conditions. This model was developed based on the evaluation of the dynamics of root infections (horizontal and vertical growth) of avocado plants and seedlings according to the measures previously reported. The algorithm implemented in Rootbox (Leitner *et al.*, 2010) was used and run under the MatLab interface (version 15.0).

Physiological response of avocado seedlings to the causal agents and disorder associated with AWC

The physiological responses of Hass avocado seedlings to the infection with *P. cinnamomi*, *V. dahliae* and the hypoxia-anoxia disorder were determined for each of the stages of disease development, according to the evaluation

scale proposed for the seedlings (Tabs. 1-3). Net photosynthesis (A), stomatal conductance (gs), and transpiration (E) were directly quantified using a TPS-2 Portable Infrared Analyzer (PhotosynThesis System®, Washington, USA). From the data obtained, water efficiency (WUE) was estimated (WUE=A/E). The quantification of the physiological variables in each of the stages of the scale was performed at the same hour in the morning (9-10 am) and on the same leaf (third fully developed leaf from the apical bud).

A completely randomized design was used with each experimental unit consisting of five plants with three replicates. Quantification was performed for five different points on the same leaf and on the five different plants of each experimental unit. Tests were performed during a 30-day period. Data were quantified each day. Data homoscedasticity and normality were verified using the criteria of Levene and Kolmogorov-Smirnov. Subsequently, data were subjected to an analysis of variance and the means were compared by the Tukey test with a significance of 95% (*P*≤0.05). The analysis was run on the free software R (R Development Core Team, 2019).

Results and discussion

Development and calibration of scales for principal pathogens and disorder associated with AWC under field and net house conditions

For the disease development scale of *P. cinnamomi* in the field, the following five stages were determined: Stages 1 and 2 were characterized by leaf yellowing and the absence of active growing buds, leading to stunted growth for stages 3 and 4. The previous symptomatology became more pronounced with concurrent generalized wilt, besides defoliation. For stage 5, plant death or irreversibly damaged leading to death occurred due to defoliation and the presence of dieback. Each of the stages of disease development beginning from stage 1 was characterized by an increase in the number of necrotic secondary and tertiary rootlets as a consequence of *P. cinnamomi* colonization (Tab. 1, Fig. 1).

For *V. dahliae,* the scale of disease development in the field during stages 1 and 2 was characterized by slight yellowing and wilting on one side of the plant. For stages 3 and 4, plant growth stopped, wilt intensified, the leaves became brown and adhered to the plant, and there was the presence of stem necrosis. Root necrosis occurred increasingly in stages 1, 2 and 3 and for the remaining stages it remained constant. In addition, root necrosis was observed in the main, secondary and tertiary roots (Tab. 2, Fig. 2).

In the case of the abiotic disorder originated by hypoxia-anoxia, the first stages of the disease (1 and 2) showed similar symptoms to those caused by *P. cinnamomi* and *V. dahliae* that were characterized by foliar yellowing. For the other stages (3 and 4), there was generalized wilt and plant defoliation. For the root system, a gradual increase of the necrosis as the scale increased was observed in a similar way as for *P. cinnamomi*. However, the difference for hypoxia-anoxia was that this necrosis was generalized in all roots (primary, secondary and tertiary) and the internal color was purple (Tab. 3, Fig. 3).

TABLE 1. Description of the disease developmental scale for *Phytophthora cinnamomi* in avocado cv. Hass grafted on West Indian rootstock and evaluated under field (a) and net house (b) conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[a]	Visible disease symptoms. Mild leaf yellowing and lack of active growing buds that leads to stunted growth	Diseased rootlets between 10 and 15%
2[a]	Pronounced leaf yellowing and stunted growth	Diseased rootlets between 15.1 and 25%
3[a]	Generalized leaf yellowing, wilting and mild defoliation <35%	Diseased rootlets between 25.1 and 50%
4[a]	Generalized leaf yellowing, wilting and defoliation between 35.1 and 90%	Diseased rootlets between 50.1 and 90%
5[a]	Irreversible damage to death, dieback and severe defoliation >90.1%	Diseased rootlets >90%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Generalized leaf yellowing	Diseased rootlets between 10 and 15%
2[b]	Generalized leaf yellowing, stunted growth and mild wilting	Diseased rootlets between 15.1 and 70%
3[b]	Generalized leaf yellowing and wilting, defoliation	Diseased rootlets >70.1%

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

Agron. Colomb. 38(1) 2020

89
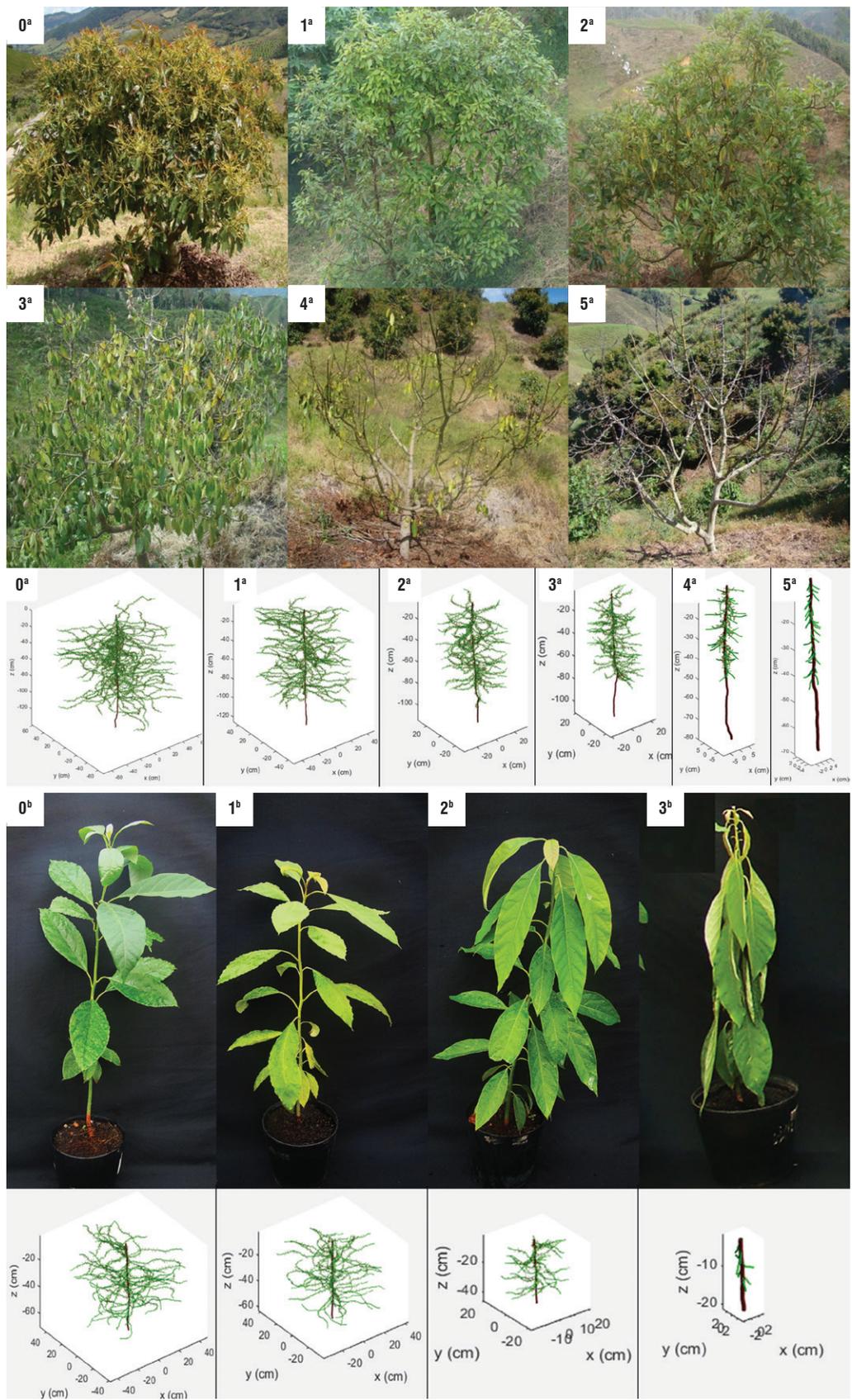
FIGURE 1. Visual and root appearance of disease developmental scale for *Phytophthora cinnamomi* in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific description is presented in Table 1.
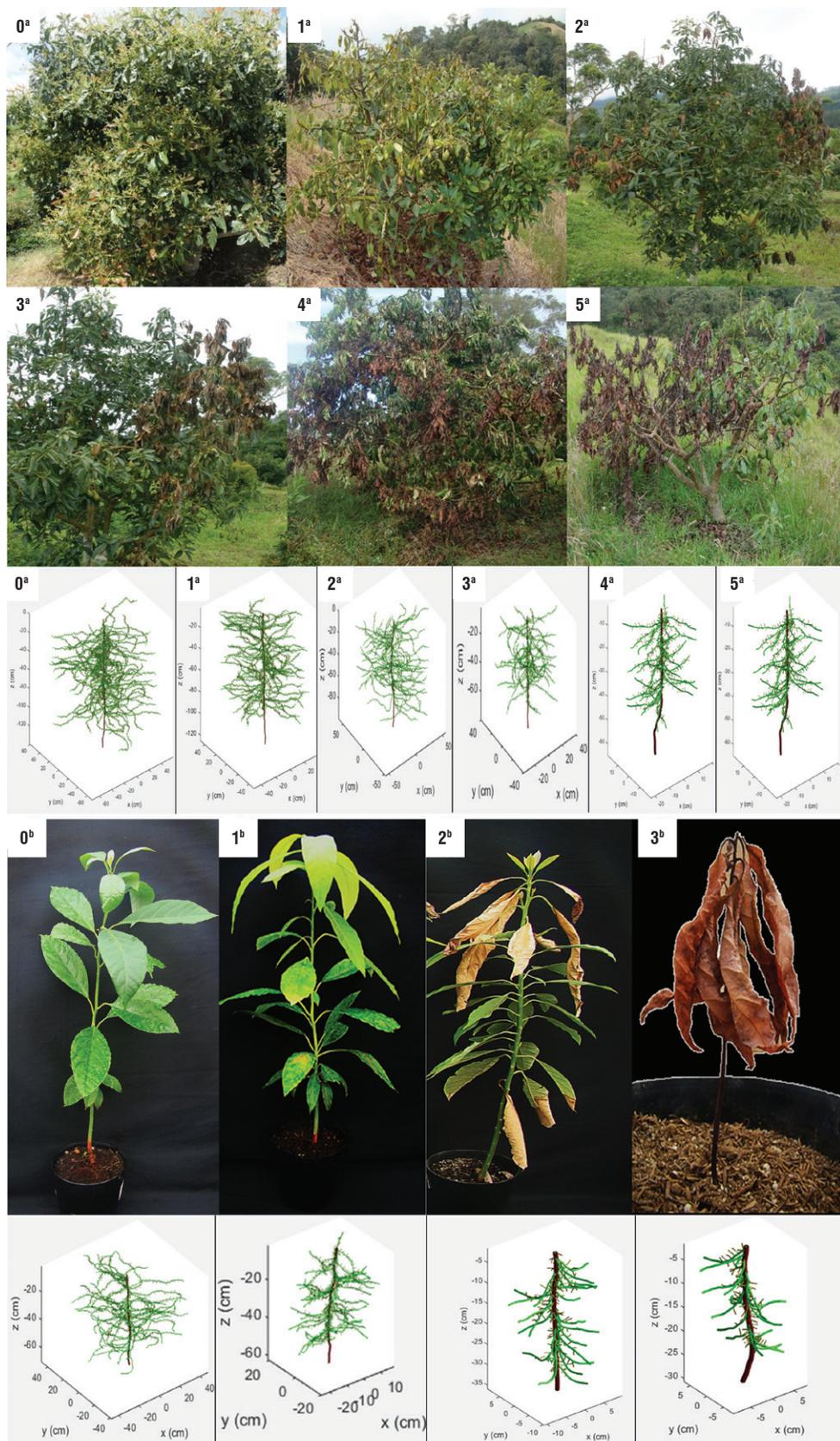
FIGURE 2. Visual and root appearance of disease developmental scale for *Verticillium dahliae* in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific descriptions are found in Table 2.

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

91

TABLE 2. Description of disease developmental scale for *Verticillium dahliae* in avocado cv. Hass grafted on West Indian rootstock evaluated under field (a) and net house (b) conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[a]	Visible disease symptoms. Mild leaf yellowing	Necrotic roots between 10.1 and 20%
2[a]	Pronounced unilateral leaf yellowing, mild wilting and stunted growth	Necrotic roots between 20.1 and 25%
3[a]	Pronounced unilateral wilting with leaf death <35%. Leaves remain adhered to the stem	Necrotic roots between 25.1 and 30%
4[a]	Unilateral wilting with leaf death between 35.1 and 70% in the affected portion of the tree. Stem necrosis in the affected portion of the tree	Necrotic roots 30.1 and 40%
5[a]	Irreversible damage to death. Stem and leaf death >70.1%	Necrotic roots<40%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Generalized leaf yellowing	Necrotic roots between 10.1 and 20%
2[b]	Leaf death <50%	Necrotic roots 20.1 and 30%
3[b]	Collapse of the seedling, necrotic brown leaves that remain adhered to the stem	Necrotic roots <40%

Under net house conditions, the disease developmental scales showed a similar trend of symptoms in the shoot and in the number of roots affected when compared to what was found for field conditions (Tabs. 1-3, Figs. 1-3). Stages 4 and 5 were not included for seedlings grown under net house conditions. This was because in stage 3, the seedlings infected with *P. cinnamomi* and affected by hypoxia-anoxia already showed a large number of affected roots, and for *V. dahliae* they showed stem necrosis. In stage 3 of the development of the disease, plant death induced by all three causal agents analyzed was already irreversible.

Symptom expression in the shoot of the plants was similar to the three pathogens and the disorder evaluated during the first stage of disease development, mainly characterized by foliar yellowing and wilting. However, clear differentiation of symptoms induced by each causal agent and disorder was observed after stage 2 of the disease's development. *Phytophthora cinnamomi* induced generalized wilting, partial or massive defoliation, dieback and necrosis of secondary feeder roots. In contrast, wilt induced by *V. dahliae* caused generalized ringing and leaf necrosis that remained adhered to the stems. In addition, hypoxia-anoxia caused foliar wilting and defoliation (Figs. 1-3).

AWC symptomatology has been widely reported and explained because the root system is affected, leading to a reduction in nutrient and water uptake from the soil. This induces decompensation resulting from water loss that gives rise to the expression of wilting symptoms in the shoot such as yellowing, scarce foliage, absence of actively growing buds causing stunted growth and wilt (Bingham and Zentmyer, 1954; Zentmyer, 1984; Whiley *et al.*, 1987). Similarly, it is reported that conditions of moisture excess in the soil lead to root decay, and as a consequence such conditions decrease the assimilation and translocation of nitrogen (Stolzy *et al.*, 1967; Ezin *et al.*, 2010). Multiple morphological and physiological changes that make growth stop also accelerate senescence and modulate other physiological processes (Wager, 1942; Stolzy *et al.*, 1967;

TABLE 3. Description of disease developmental scale for hypoxia-anoxia in avocado cv. Hass grafted on West Indian rootstock evaluated under field conditions.

Scale value	Appearance of the shoot and disease symptoms	Appearance of roots
0[a]	Healthy plants with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable roots without affected stems
1[a]	Visible disease symptoms. Mild leaf yellowing and lack of active growing buds	Necrotic roots between 10.1 and 20%
2[a]	Generalized and pronounced yellowing	Necrotic roots between 20.1 and 40%
3[a]	Generalized wilting with fast defoliation <30%	Necrotic roots between 40.1 and 70%
4[a]	Severe wilting and defoliation between 30.1 and 70%	Necrotic roots>70.1%
5[a]	Irreversible damage to death, defoliation >70.1%	Necrotic roots>90.1%
0[b]	Healthy seedlings with abundant foliage of dark green color and foliar buds in active growth. No disease symptoms	>90% of viable rootlets
1[b]	Visible disease symptoms. Mild leaf yellowing and wilting. Lack of active growing buds	Necrotic roots between 10.1 and 20%
2[b]	Generalized yellowing and pronounced wilting	Necrotic roots between 20.1 and 70%
3[b]	Severe defoliation, dead seedling or irreversible damage resulting in death.	Necrotic roots >70%.
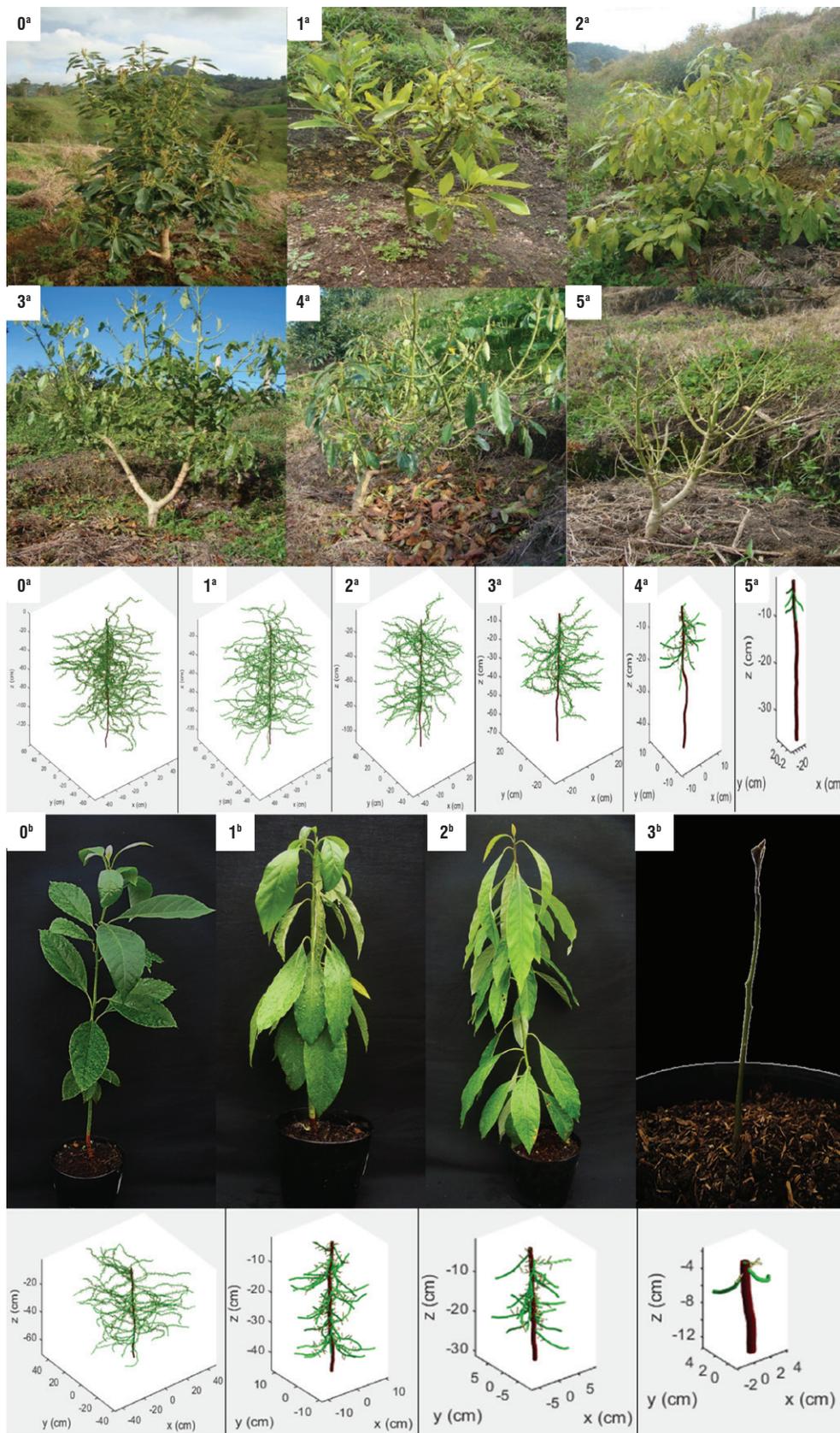
FIGURE 3. Visual and root appearance of disease development scale for hypoxia-anoxia in avocado cv. Hass grafted on West Indian rootstock evaluated under (a) field and (b) net house conditions. Specific description is presented in Table 3.

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

93

Sanclemente *et al.*, 2014). For the more advanced stages of these evaluated diseases, characteristic expressions of each pathology were observed. *Phytophthora cinnamomi* colonizes and destroys large portions of the root system, almost exclusively limited to secondary and tertiary roots, confirming previous reports (Zentmyer, 1984; Ramírez-Gil and Morales-Osorio, 2019). On the other hand, *V. dahliae* affects the roots and later the mycelium invades the vascular tissues, especially the xylem. From there, it begins its upward movement producing abundant reproductive structures when invading the plant, causing typical unilateral wilting symptoms in the branches (Zentmyer, 1949, 1984). For hypoxia-anoxia, the most marked characteristics are rapid defoliation, reduction of growth, and necrosis of the whole root system (Stolzy *et al.*, 1967; Sanclemente *et al.*, 2014).

For the *P. cinnamomi* evaluation under field conditions, the regression model that established the relationship between the amount of inoculum and the value of the scale showed a correlation coefficient of 0.95, a coefficient of determination of 91.3%, and a highly significant ($P<0.000$) and lower value in the AIC. For the other two diseases, the statistical parameters of correlation and determination coefficients were 0.94 and 89.0% for *V. dahliae* and 0.97 and 93.8% for hypoxia-anoxia. They were highly significant ($P<0.000$) and with lower values in the AIC (Fig. 4). For the relationships between inoculum quantity of *P. cinnamomi*, *V. dahliae* and necrotic roots associated with hypoxia-anoxia, the values of the disease scales developed for seedlings under net house conditions were highly significant ($P<0.000$), with correlation and determination coefficients of 0.92-85.3, 0.97-95 and 0.84-71.3% (Fig. 4).

The inoculum from soil and plant tissues and necrotic roots and their corresponding values of disease development scale showed a significant ($P<0.05$) and positive relationships for the three causal agents tested, confirming that these variables are correlated. This result may be useful for estimating theoretical inoculum and progress in values of affected tissues for both field and net house conditions. Precision using the disease scales designed in the present work is likely to be high. This calibration assumes that the descriptive and arbitrary definition of these scales was adequate since good representation of the dynamics of these two diseases and disorder were obtained. This provides a tool for the study of these pathologies, and allows the use of a different scale for each of these causal agents.

Most of the scales developed to date have been designed for *P. cinnamomi* (Darvas *et al.*, 1984; Coffey, 1991), but in

this research, a contribution has been made by the development of specific scales for the avocado wilt disease complex caused by *P. cinnamomi*, *V. dahliae,* and hypoxia-anoxia disorder, both in the roots and shoots of the plants under net house and field conditions during the dry and the rainy seasons. Additionally, the regression analysis showed a high relationship between the scale and the inoculum and necrotic root values.

Evolution through time and the stages of disease development caused by pathogens and disorder associated with AWC

Dynamics through time of the scales of disease development evaluated under field conditions for *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia showed that both pathogens and the disorder caused plant death in a shorter period of time during the rainy season. Hypoxia-anoxia was the fastest developing condition with a mean time of 71.3 d, followed by *P. cinnamomi* (150.5 d), and *V. dahliae* (185.4 d). During the dry season *P. cinnamomi* caused plant death at 205.5 d and *V. dahliae*, took a longer period of time (stage 5) of 215.4 d (Supplementary material 3A and B). As expected, during the dry season no plant deaths by hypoxia-anoxia were recorded. Similarly, for the evaluation under net house conditions, the disease that more rapidly caused seedling death (stage 3) was hypoxia-anoxia (78.4 d) followed by *P. cinnamomi* (115.4 d), and *V. dahliae* (170.5 d) (Supplementary material 3A and B).

The inoculum values and necrotic roots showed a rapid increase ($P<0.05$) during the first stages (1-3) of the scale of disease development. After stage 3 (stages 4 and 5, plant death) of the disease development scale, the rate of increase of inoculum was slower compared to stages 1, 2 and 3. Variation in the inoculum values showed a similar pattern under field and net house conditions (Supplementary material 3 C-F).

The results associated with the dynamics of the disease in the field confirm that the rainy season accelerates the physiopathological processes associated with the diseases' development for the two causal agents and the particular disorder that we investigated. Soil moisture is considered one of the most important factors in the dynamics of the avocado wilt complex. This depends on the soil's physical and chemical properties, precipitation patterns, water interactions in the sub-surface horizons of the soil and the congruence of drainage networks (Ramírez-Gil, 2018; Ramírez-Gil and Morales-Osorio, 2018). Soil moisture has been widely reported as the most important factor in the
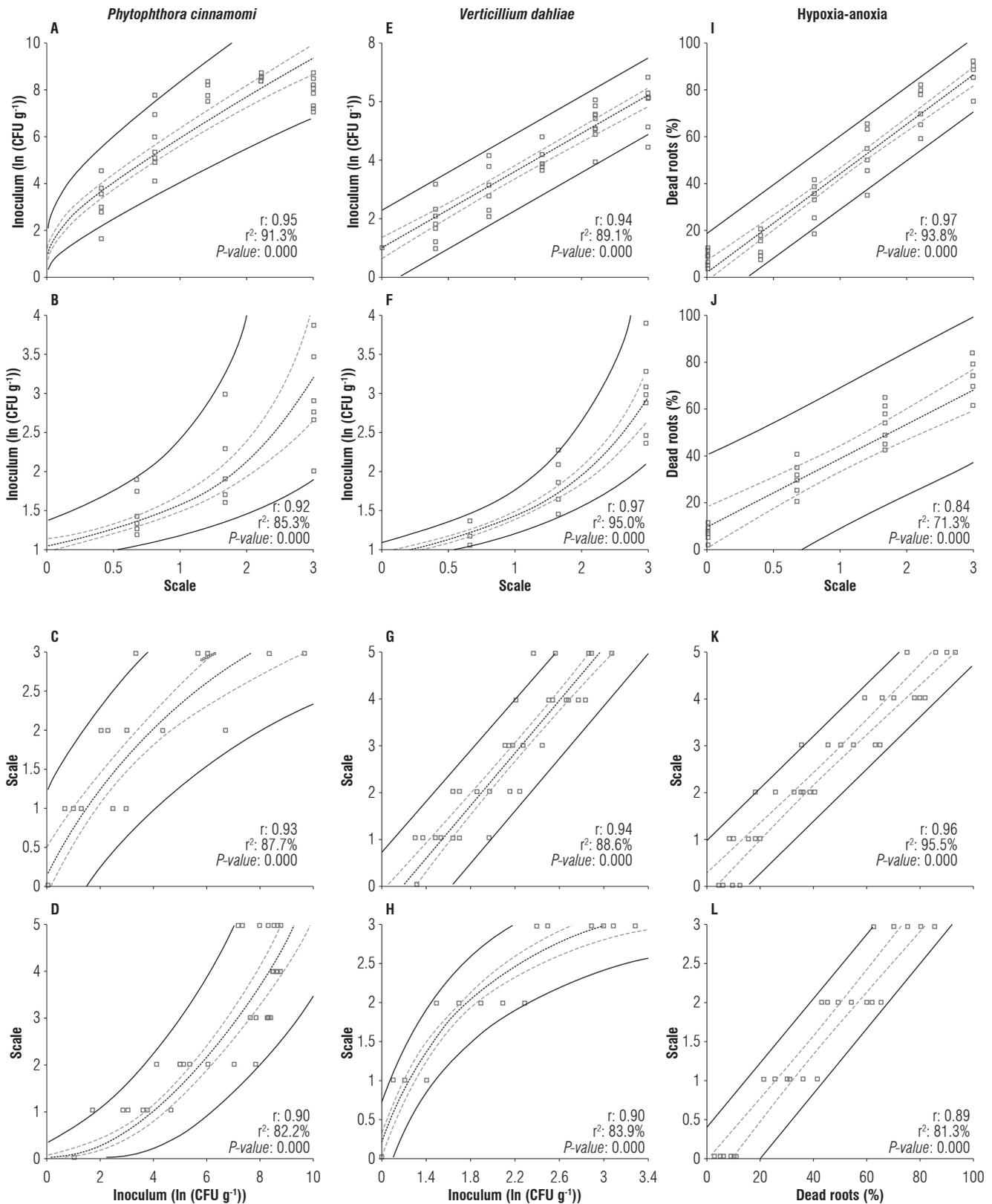
FIGURE 4. Models obtained for relationships between the amount of inoculum and the scale of disease development in *Phytophthora cinnamomi*, *Verticillium dahliae*, and conditions of hypoxia and anoxia under field and net house conditions. A, C, E, G, K, models developed under field conditions data and I, B, D, F, H, J and L models developed under net house conditions. Models were selected based on the correlation and determination coefficient, significance (*P*<0.001). A, B, E, F, I, and J inoculum in function of scale and C, D, G, H, K, and L, scale in function of inoculum.

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*,
Verticillium dahliae and hypoxia-anoxia disorder and their physiological responses in avocado plants

95

development of the life cycle of *P. cinnamomi*. Free water is of crucial importance in the dispersion of the inoculum source (Sterne *et al.*, 1977; Gisi *et al.*, 1980; Coffey, 1991; Ramírez-Gil and Morales-Osorio, 2018). During the dry season we also observed the symptomatology induced by *P. cinnamomi* and *V. dahliae*. Although the amount of inoculum produced was possibly lower under these conditions (Ramírez-Gil and Morales-Osorio, 2018), the soil water deficit and the affected root system made it difficult for the plant to uptake nutrients and water, and this leads to a rapid and marked expression of symptoms.

Physiological responses to the principal pathogens and disorder associated with AWC

The physiological responses of Hass avocado seedlings markedly changed according to the stage of disease development caused by *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia. Net photosynthesis showed a significant decrease ($P<0.05$) for *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia in stage 3 (irreversibly damaged to death) with values of 7.5, 9.1 and 87.2%, respectively, compared to stage 0 (healthy plants) (Fig. 5).

Stomatal conductance and transpiration showed similar behavior to net photosynthesis ($P<0.05$) (Fig. 5). Hypoxia-anoxia induces a maximum reduction of stomatal conductance and transpiration values at stage 2, so no further change was observed in stage 3. Similarly, for *P. cinnamomi* and *V. dahliae*, the values observed for the same variable decreased as the disease scale values increased during all stages of disease development. The indirect variable water use efficiency (Fig. 5) did not show significant differences ($P>0.05$) during the stages 0 and 1 of disease development for all three causal agents of avocado wilt tested. For stages 2 and 3, this parameter significantly increased indicating a more efficient use of water in these stages in the plants affected by *P. cinnamomi*, *V. dahliae*, and hypoxia-anoxia, compared to the plants in stage 1 and healthy plants (stage 0) ($P<0.05$).

The decrease in the values of physiological variables, such as net photosynthesis, stomatal conductance and transpiration, is the plant's response to a source of stress caused by these pathologies, and this becomes more noticeable with the progression of the disease. This condition occurs as a
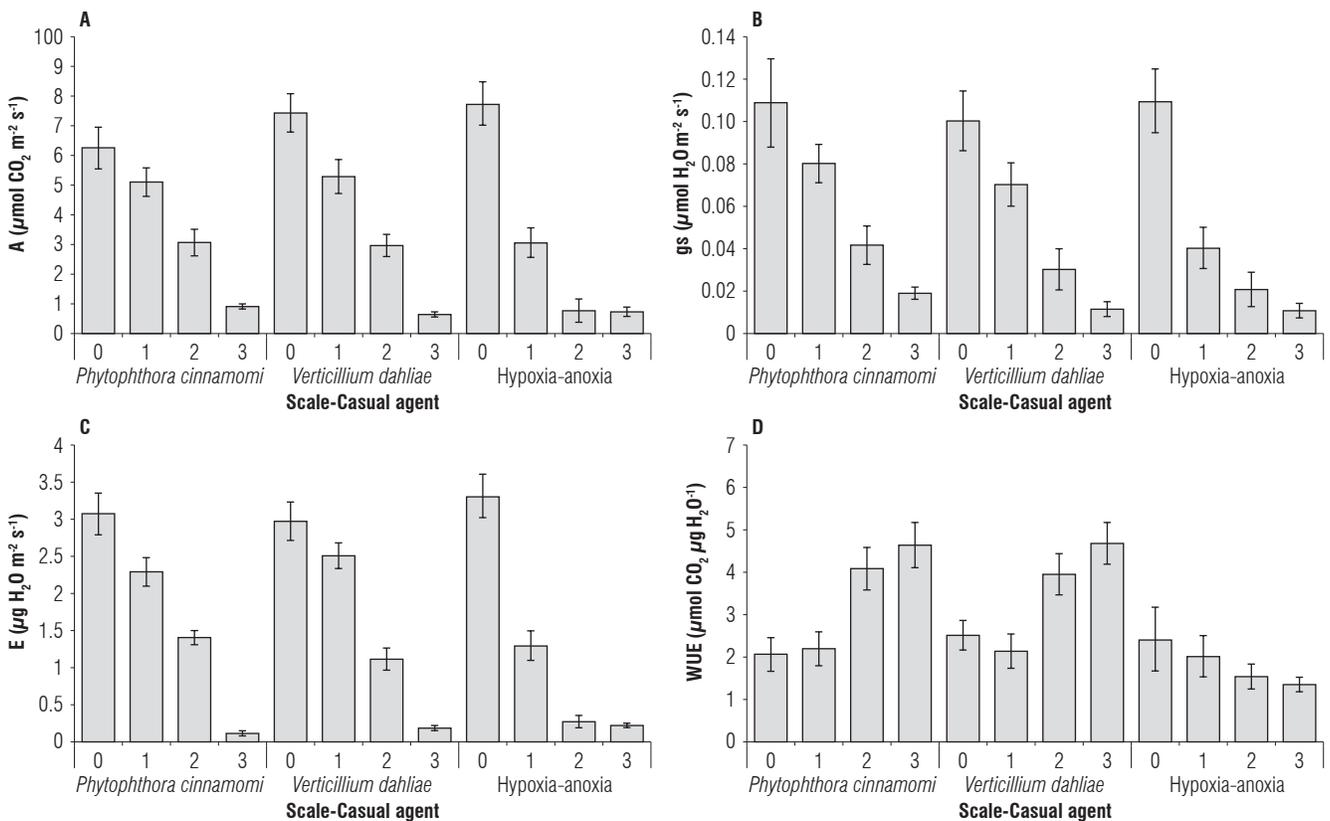
FIGURE 5. Physiological responses of avocado seedlings cv. Hass to *Phytophthora cinnamomi*, *Verticillium dahliae*, and hypoxia and anoxia causing the avocado wilt complex. (A) Net photosynthesis, (B) stomatal conductance, (C) transpiration, (D) water use efficiency. Error bars represent the confidence intervals of the means, validated by the Tukey mean separation test. No overlapping of the error bars indicates significant differences ($P<0.05$).

result of affected plants having many damaged roots and tissues, which reduces the uptake and transport of water in the plant and leads to a water deficit, as occurs for plants infected by *P. cinnamomi* and *V. dahliae* or due to the hypoxia-anoxia disorder.

For these conditions, the physiological responses of avocado seedlings were very similar, and were a little more marked with an excess of humidity, especially during the first stages (Fig. 5). The main mechanism involved that explains the response of the plant is considered to be stomatal closure (Sanclemente *et al.*, 2014), since this condition indirectly affects stomatal conductance, net photosynthesis and transpiration (CuiYing *et al.*, 2010; Yin *et al.*, 2010).

Under a specific stress condition, such as a hypoxia-anoxia or infection by a pathogen, a plant may have adaptive responses, which could be morphological, anatomical or physiological (CuiYing *et al.*, 2010). However, if the stressful condition continues and increases, the plant cannot overcome it, leading inevitably to great damage or death as observed in stage 3 of disease development for each one of the causal agents studied in the present research (Ploetz and Schaffer, 1989; Reeksting *et al.*, 2014).

In studies of avocado, under conditions of hypoxia-anoxia and the interaction with *P. cinnamomi* there is a decrease in photosynthesis that is accompanied by a reduction in stomatal conductance, transpiration and partial pressure of CO_2 in the intercellular leaf spaces (Ploetz and Schaffer, 1989; Reeksting *et al.*, 2014). These results agree with what was found in this research.

Greater water use efficiency occurred in the more advanced stages of the disease. The plant under this condition attempts to maximize the use of each one of the resources that it possesses, especially water, since water deficit in plant tissues becomes elevated when plants have a very reduced and atrophied root system.

Conclusion

Scales developed to evaluate the progress of wilt disease in avocado cv. Hass in field and net house conditions are highly correlated with the amount of inoculum in each of the defined stages. Therefore, they are adequate for evaluating the dynamics through time of each of these pathologies. Avocado seedlings are highly affected by the presence of these pathologies, especially in the late stages close to death, as seen by the physiological responses measured.

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Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

97

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SUPPLEMENTARY MATERIAL 1. Edaphic variables associated with soil in lots and laboratory tests.

Soil	Sn[1]	Si[1]	Cla[1]	pH	OM[2]	Al[3]	Ca[3]	Mg[3]	K[3]	P[4]	S[3]	Cu[5]	Zn[5]	Mg[5]	B[5]
Donmatias[a]	52	28	20	5,2	12.3	0.6	2.55	1.1	0.45	21.3	5.1	0.8	2.3	1.9	0.3
El Retiro[b]	60	22	18	5.3	14.8	0.8	3.5	1.3	0.55	24.4	5.6	0.9	1.9	2.5	0.5
La ceja[c]	58	23	19	5.5	13.1	1.6	1.9	0.9	0.35	15.9	5.9	0.9	2.4	2.1	0.35
Net house[c]	62	15	23	5.1	6.0	0.9	1.1	0.9	0.4	12.3	4.8	0.6	1.7	1.9	0.2

[1]Sand (Sn, %), silt (%), clay (Cla, %) (Bouyoucos). pH (water:soil, 1:2, V:V). [2]Organic matter content (OM, %) (Walkley and Black; aluminium). Aluminium (Al) (1M KCl). Calcium (Ca), magnesium (Mg) and potassium (K) (1M ammonium acetate). Phosphorus (P) (Bray II). Sulfur (S) (0.008 M calcium phosphate solution). Iron (Fe), manganese (Mn), copper (Cu) and zinc (Zn (Olsen-EDTA buffer). Boron (B) (hot water). [3]Interchangeable bases (cmolc kg^{-1}). [4]mg kg^{-1}. [5]Available minor elements (mg kg^{-1}). [a]The plot was located in the Municipality of Donmatias (6.496961 latitude, 75.412118 longitude, 2213 m a.s.l.) in the Northern region of Antioquia (Colombia). [b,c]The plots were located in the municipalities of El Retiro (6.09715 latitude, 75.46478 longitude, 2100 m a.s.l.) and La Ceja (5.95931 latitude, 75.41777 longitude, 2387 m a.s.l.), in the eastern region of Antioquia (Colombia). All plots were in a life zone classified as tropical lower montane wet forest (TLM-wf) *sensu* Holdridge (Holdridge, 1967). [c]Universidad Nacional de Colombia Medellin campus (6°15' N, 75°34' W, 1496 m a.s.l), with an average temperature range of 18-22°C, relative humidity in the range of 75-95% and a photosynthetically active radiation of 650-1920 μmol photons m^{-2} s^{-1}.
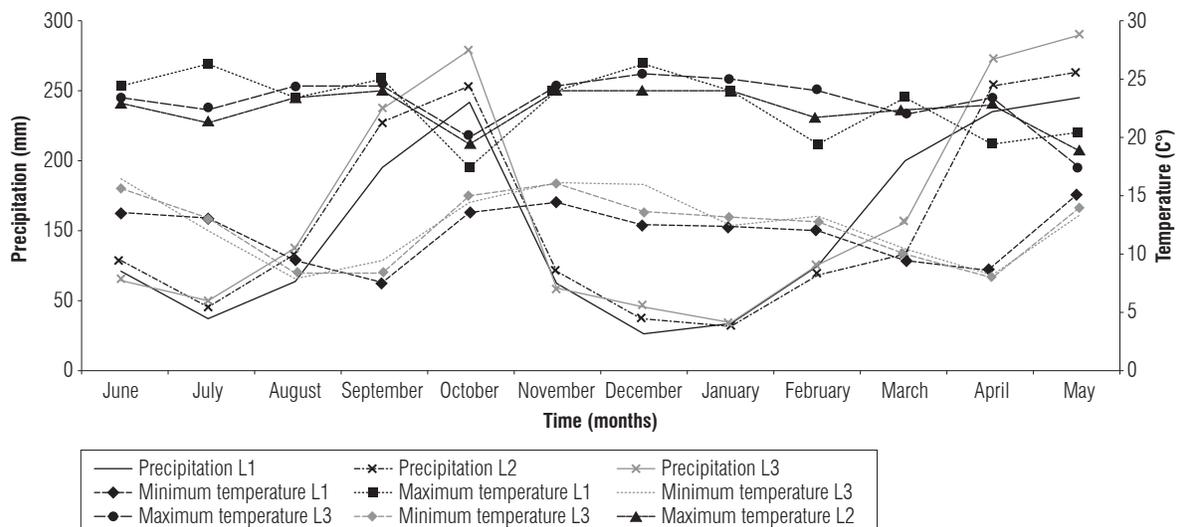
SUPPLEMENTARY MATERIAL 2. Climatic conditions associated to the lots evaluated in the two study regions. L1: Lot in Donmatias. L2: Lot in El Retiro. L3: Lot in La Ceja. Climate values represent the average for each month during the years 2011-2012.

Ramírez-Gil and Morales-Osorio: Development and validation of severity scales of avocado wilt complex caused by *Phytophthora cinnamomi*, *Verticillium dahliae* and hypoxia-anoxia disorder and their physiological responses in avocado plants

99
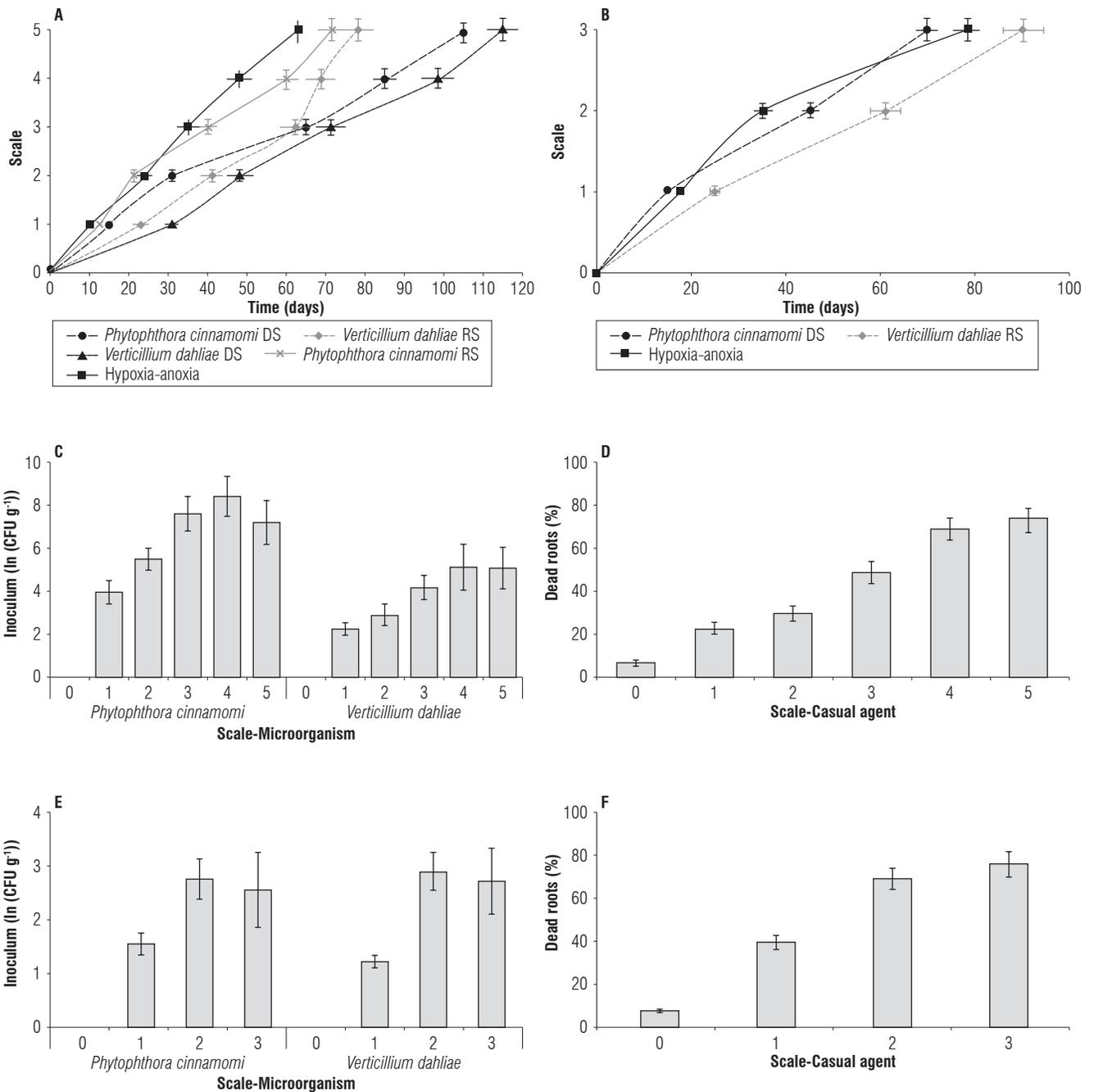
SUPPLEMENTARY MATERIAL 3. Variation through time of the disease development scales and inoculum values for *Phytophthora cinnamomi*, *Verticillium dahliae,* and hypoxia and anoxia in avocado cv. Hass evaluated under field and net house conditions. (A) Variation through time of the disease development scale under field conditions. (B) Variation through time of the disease development scale under net house conditions. (C) Variation through time of the inocula values for *Phytophthora cinnamomi* and *Verticillium dahliae* under field conditions. (D) Variation through time of the hypoxia and anoxia scale values under field conditions. (E) Variation through time of the inocula values for *Phytophthora cinnamomi* and *Verticillium dahliae* under net house conditions. (F) Variation through time of hypoxia and anoxia scale values under net house conditions.