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Plant protection

Aggressiveness of *Phytophthora cinnamomi* in avocado seedlings and effect of pathogen inoculum concentration and substrate flooding on root rot and development of the plants

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Abstract - The present study evaluated the aggressiveness of *Phytophthora cinnamomi* isolates and the effect of pathogen inoculum concentration and periods of substrate flooding on root rot and plant development. Twelve pathogen isolates were inoculated on the collar region of avocado seedlings with or without wounding. Only 31.3% of the inoculated plants without wounding developed lesions, compared to 100% of the plants with wounding, while the isolates showed different aggressiveness levels. Avocado seedlings had their substrate inoculated with 0, 0.1% and 1.0% (m/v) wheat seeds colonized by the pathogen per pot, and four periods of 0, 12 and 24 h substrate flooding were produced at fortnightly intervals. The assessed parameters were number of leaves per plant, collar diameter, plant height, leaf area index, visual severity percentage of infected roots, fresh mass (%) of diseased roots and dry mass of shoot and roots. Both pathogen inoculation and substrate flooding caused root rot; however, combination of these two factors produced an additional effect on disease symptoms. Root rot severity was superior to 50% when soilless substrate had 0.1% (m/v) *P. cinnamomi* inoculum and flooded for 12-24 h after inoculation, conditions that can be recommended for pathogenicity and disease control studies using potted avocado plants.

Index terms: *Persea americana*, root disease, soil flooding.

Agressividade de *Phytophthora cinnamomi* em mudas e abacateiro e efeito de concentrações de inóculo do patógeno períodos de inundação na podridão de raízes e no desenvolvimento das plantas

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Resumo - Avaliou-se a agressividade de isolados de *Phytophthora cinnamomi* em abacateiro e estudou-se o efeito de concentrações de inóculo do patógeno e períodos de inundação do substrato na podridão radicular e no desenvolvimento das plantas. A inoculação de 12 isolados do patógeno foi realizada no colo de mudas de abacateiro, com ou sem ferimento. Apenas 31,3% das plantas inoculadas sem ferimento desenvolveram lesões, comparado a 100% das plantas com ferimento, com diferenças entre os isolados quanto à agressividade. Mudas de abacateiro tiveram o substrato inoculado com 0; 0,1 e 1,0% (m/v) de sementes de trigo colonizadas pelo patógeno por vaso, sendo submetidos a quatro períodos de inundação do substrato em intervalos quinzenais, a saber: 0, 12 e 24 horas. Avaliou-se o número de folhas por planta, o diâmetro do colo, a altura da planta, o índice de área foliar, a severidade visual (%) de raízes doentes, a porcentagem de massa fresca de raízes doentes e a massa seca da parte aérea e das raízes. Tanto a inoculação do patógeno como a inundação do substrato causaram podridão radicular, contudo, a combinação destes dois fatores resultou em um efeito aditivo nos sintomas da doença. Períodos de inundação de 12-24 h após a infestação do substrato com 0,1% (m/v) de inóculo de *P. cinnamomi* provocaram severidade superior a 50% de podridão radicular, podendo ser recomendados em estudos de patogenicidade e de controle da doença em plantas envasadas de abacateiro.

Termos para indexação: *Persea americana*, doença radicular, saturação do solo.

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Introduction

One of the major diseases affecting avocado plants (*Persea americana* Miller) worldwide, both in the nursery and in the field, is Phytophthora root rot (PRR). Several *Phytophthora* species can cause the disease, with *Phytophthora cinnamomi* Rands being the most important of them and the most widespread in the avocado producing regions of the world (DANN et al., 2013). In Brazil, *P. cinnamomi* is the species usually associated with the disease (SUMIDA et al., 2009; PICCININ et al., 2016, RODRIGUEZ, 2016). In the State of São Paulo, Brazil, *P. cinnamomi* mating type A2 was the only species found so far associated with the disease, as confirmed by surveys carried out in avocado nurseries and commercial orchards located in 17 municipalities in the state (FEICHTENBERGER; SILVA, personal communication). PRR is a disease of fine feeder roots that impacts plant productivity and longevity. Infected feeder roots become brittle and turning black as the root tissue decay, which restricts water and nutrient uptake by the tree that leads to wilting, defoliation and branch-dieback, production of smaller fruits, and eventual tree death (REEKSTING et al., 2014; PICCININ et al., 2016). *P. cinnamomi* occasionally invades larger roots and sometimes causes a weeping canker at the base of the tree that may extend up the trunk for at least 1 m. These trunk cankers originate at, or just below, the soil line (DANN et al., 2013).

High disease incidence and severity are frequently associated with poorly drained clayish soils subject to long flooding periods (DANN et al., 2013). Motile pathogenic spores, called zoospores, are produced and released from sporangia onto the surface of soil or substrate in the presence of water, being capable of infecting the roots. Besides high soil humidity, the disease depends on temperatures between 21 and 30°C to occur (PICCININ et al., 2016). In addition, soil flooding may damage the roots due to anoxia, predisposing plants to infection by the pathogen (BESOAIN et al., 2005). Avocado trees are sensitive to flooding, and plants with poor growth and yield, symptoms of nutrient deficiencies, branch-dieback, and even tree death may occur in flooded or poorly drained soils (SCHAFFER et al., 2013).

Recommended measures for the management of this disease include using healthy seedlings planted in deep and well-drained soils and adopting appropriate irrigation to prevent prolonged flooding periods (PICCININ et al., 2016). Leaf spraying with potassium phosphite, combined or not with dolomitic limestone or plaster applied to the soil, allows partial recovery of diseased avocado plants (SILVA et al., 2016). Phosphites can also be injected into the stem of plants for disease control. However, scientific studies on the effectiveness of root rot control methods in avocado trees are relatively scarce in Brazil, mainly due to difficulties in reproducing disease symptoms in plants inoculated with the pathogen.

Based on the need of investigation to standardize pathogen inoculation methodologies, the present study aimed to evaluate the effect of substrate flooding periods and pathogen inoculum concentration on the disease development in 'Hass' and 'Margarida' avocado seedlings, as well as knowing the aggressiveness of *P. cinnamomi* isolates from different producing regions in São Paulo State, Brazil, in 'Margarida' avocado seedlings.

Material and Methods

Experiments were conducted in 2018 at the facilities of the São Paulo Agency for Agribusiness Technology (APTA), Central-West Regional Center - Bauru, São Paulo State, Brazil (22º20'37"S and 49º03'12"W; 615 m altitude), under greenhouse conditions and controlled maximum temperature at 30ºC.

The plants used in the experiments were non-grafted seedlings originated from 'Margarida' and 'Hass' avocado seeds, supplied by "Campo de Ouro" and "Jaguacy" farms, located in Piraju and Bauru municipalities São Paulo State, respectively.

Twelve *P. cinnamomi* isolates of the sexual compatibility group A2 were employed: LRS 06/08, LRS 191/10, LRS 01/11, LRS 02/11, LRS 14/11, LRS 15/11, LRS 17/11, LRS 18/11, LRS 69/11, LRS 133/11, LRS 05/12 and LRS 06/12. They were supplied by the Mycological Culture Collection "Dra. Victoria Rossetti" of the Instituto Biológico (IB), at the Regional Laboratory of Sorocaba (LRS/IB/APTA), and had been isolated from 2008 to 2012, from avocado plants showing typical PRR symptoms, of the cultivars Breda, Fortuna, Hass and Margarida, in commercial orchards located in six municipalities in the State of São Paulo: Bauru, Bernardino de Campos, Itajobi, Limeira, Severínia and Taquarivaí.

Aggressiveness of *Phytophthora cinnamomi* isolates in avocado seedlings

Inoculum of each one of the 12 evaluated *P. cinnamomi* isolates consisted of 5 mm-diameter mycelial disks removed from the edges of colonies grown for ten days at 25 ºC on Petri plates (9 mm diameter) containing potato-dextrose-agar medium (PDA).

Approximately three months after sowing, 'Margarida' avocado rootstock seedlings were inoculated with a mycelial disk of the pathogen, which was attached with adhesive tape to the collar region of the plants, at a height of 2 cm from the substrate surface. The inoculation site was previously wounded or not with a histological needle at 2 mm depth. For the non-inoculated control, PDA disks without the pathogen were used.

Lesion length in the collar of plants was visually

assessed with a ruler, at 3 − 4 days intervals, during 50 days, when the lesions no longer increased in size in two subsequent evaluations. After measuring the length of the external lesions, the stem region was longitudinally opened with a switchblade to assess the length of the internal lesions. Data on external lesion in a 50 -day period were used to calculate the area under the disease progress curve (AUDPC).

Once lesion evaluation was finalized, or the plants have died, the evaluated isolates were re-isolated from the lesions to confirm them as the causal agents of the disease. This process consisted in disinfesting lesion fragments from the plant collar with sodium hypochlorite at 0.5%, for one minute, followed by washing with sterile distilled water, dividing them into smaller fragments and plating the small fragments on agar-water media (AW).

Experimental design was in randomized blocks with four replicates, and one plot was represented by one plant. Severity data were transformed into □x and underwent parametric analysis. Means were compared according to Tukey's test (p = 0.05), using the computer program Systems for Analysis of Variance - SISVAR (FERREIRA, 2019). One of the most aggressive isolates was selected for subsequent study of the effect of substrate flooding and inoculum concentration on the disease progress and plant development.

Effect of *Phytophthora cinnamomi* inoculum concentration x flooding

Inoculum of the isolate LRS 18/11, one of the most aggressive isolates in the previous experiment, was produced in wheat seeds by adding an equal volume of distilled water to the seeds and autoclaving the mixture for two consecutive days, during 30 minutes, at 121 ºC. Twenty mycelial disks, cultured in PDA, were transferred to each bag containing 250 g autoclaved wheat seeds, incubated at 25 ºC, in the dark, during four weeks, and weekly shaken to homogenize grain colonization (LEONI; GHINI, 2003).

'Hass' and 'Margarida' avocado seedlings rootstocks, at approximately three and four months after sowing and grown in 4-L and 8-L pots, respectively, were used in the experiment. Plants of both cultivars were grown in soilless substrate basically composed of pine bark. Chemical analysis of the substrate of 'Hass' avocado seedlings, sampled before infestation with the pathogen, showed the following results: Humidity = 64%; pH = 8.1; macronutrients (g kg^{-1}): nitrogen (N) = 0.28, phosphorus (P_2O_5) = 0.22, potassium (K_2O) = 0.04, calcium (Ca) = 0.48, magnesium (Mg) = 0.17, sulfur (S) = 0.03, and micronutrients (mg kg^{-1}): boron (B) = 24, copper (Cu) = 11, iron (Fe) = 3172, manganese (Mn) = 57, zinc (Zn) = 10. For 'Margarida' avocado seedlings, chemical analysis indicated: Humidity=60%; pH = 5.7; macronutrients (g kg^{-1}): N = 0.28, P_2O_5 = 0.23, K_2O = 0.04, Ca = 0.92, Mg = 0.19, S = 0.07, and micronutrients (mg kg^{-1}): B = 24, Cu = 11, Fe = 3631, Mn = 76, Zn = 18. During the experiment, plants were fertilized twice through fertigation: 15 days after the substrate infestation with the pathogen, and 45 days after such infestation. For each fertigation, 1 g/pot of the following formula was used (%): 12 N, 5 P_2O_5, 15 K_2O, 1 Ca, 1 Mg, 5 S, 0.2 Fe, 0.2 Zn, 0.06 B, 0.08 Mn, 0.05 Cu and 0.005 Mo.

Minimum, mean and maximum temperatures in the greenhouse were weekly evaluated to calculate the averages of temperatures during the experiment, from June 11th, 2018 to August 10th, 2018.

For seedling inoculation, each pot received 0, 0.1 and 1.0% mass/volume (m/v) wheat seeds colonized by the pathogen, burying the inoculum in five equidistant holes at 5 cm depth (GALLO-LLOBET et al., 1999). Plants were daily watered to reach the substrate humidity at field capacity. One day after inoculation and at four biweekly intervals, pots were individually placed in 12-liter buckets, and flooded or not with water, keeping t the substrate during flooding e completely covered by water for periods of 12 and 24 hours.

On the day of inoculation and two months after it, the following parameters were evaluated: number of leaves per plant; collar diameter at 1cm above the substrate surface, measured with a pachymeter; plant height, obtained with a measuring tape, and leaf area index (LAI), expressed as cm^2/seedling and obtained with a leaf area integrator AccuPAR, model LP-80 PAR (Photosynthetically Active Radiation). The difference for each variable of tested plant was calculated, relative to a period of two months after inoculation. At the end of the experiment, evaluations also included visual severity (%) of diseased roots, determined by estimating the percentage of necrotic root system area; fresh mass (%) of diseased roots and dry mass (g) of shoot and roots. Dry mass was obtained after drying the materials for three days in electric oven, at 70 ºC, until constant mass was reached.

The time for occurrence of the first leaf wilting symptoms after inoculation was recorded and also the incidence of wilting or dead plants showing all dry leaves at the end of the experiment.

After plant evaluations, ten necrotic root fragments per plant were disinfested with sodium hypochlorite at 0.5%, for one minute, washed in sterile distilled water, divided into smaller fragments, which were plated onto carrot-agar selective medium, containing carbendazim (0.01 g/L), nystatin (0.025 g/L), rifampicin (0.01 g/L) and ampicillin (0.25 g/L), to confirm the presence of the root rot causal agent

Experimental design was in randomized blocks in factorial arrangement (three flooding periods x three concentrations of pathogen inoculum), including five replicates; each plot was represented by one plant. Data were transformed into □x or □x+0.5 and means were

compared according to Tukey's test (p = 0.05), using the computer program SISVAR (FERREIRA, 2019). The experiments with the two avocado cultivars were carried out simultaneously but analyzed separately. Data of the two cultivars were not compared each other because the plants were of different ages and kept in pots of different sizes, conditions that could interfere in the performance of the plants in relation to the evaluated parameters.

Results and Discussion

Aggressiveness of *Phytophthora cinnamomi* isolates in avocado seedlings

Visual symptoms of necrotic lesions in the collar region of plants previously wounded or not could be noticed from the third day of inoculation with *P. cinnamomi* isolates. Of the plants inoculated without previous wounding, only 31.3% developed symptoms and those inoculated with the isolates LRS 14/11 and LRS 133/11 did not show any symptom, which evidences the occurrence of differences in the pathogenicity of the isolates evaluated, and also the importance of the wounds for the occurrence of typical lesions of the disease. Due to the non-occurrence of the disease in most of the plants inoculated without previous wounding, the isolates were not compared with each other for aggressiveness when this inoculation methodology was used and data are not presented.

All 12 *P. cinnamomi* isolates evaluated induced typical lesions of the disease when plants were previously wounded and differences in the aggressiveness of the isolates were observed, as expressed by lesion length in the collar region of the plants (Table 1). The smallest external and internal lesions and the lowest AUDPC were obtained in plants inoculated with the isolate LRS 133/11, lesions that did not differ in length from those of the control treatment. The greatest external lesions and AUDPC were obtained in plants inoculated with the isolates LRS02/11, LRS17/11, LRS15/11, LRS06/08, LRS01/11, LRS69/11, LRS06/12, LRS14/11 and LRS18/11, while the largest internal lesions were observed in plants inoculated with the isolates LRS02/11, LRS17/11, LRS06/08, LRS01/11, LRS69/11, LRS06/12, LRS14/11 and LRS18/11. Mean length of 5cm was obtained in external lesions caused by all the *P. cinnamomi* isolates, with the exception of the isolate LRS 133/11; comparing the same lesion, the mean length of the internal lesion was 28% superior to that of the external lesion.

Table 1. Length of external and internal necrotic lesions in the collar of 'Margarida' avocado plants and Area Under the External Lesion Progress Curve (AUELPC) in plants inoculated with different *Phytophthora cinnamomi* isolates from avocado plants.

Isolates	External lesion	Internal lesion	AUELPC[1]
Control	0.1 a	0.1 a	5.0 a[2]
LRS 133/11	0.7 ab	0.7 ab	25.2 ab
LRS 05/12	3.4 bc	4.6 bc	117.2 bc
LRS 191/10	3.4 bc	4.8 bc	148.3 bc
LRS 02/11	4.6 c	7.2 c	198.6 c
LRS 17/11	5.1 c	5.9 c	199.3 c
LRS 15/11	5.2 c	5.6 bc	224.3 c
LRS 06/08	5.5 c	6.6 c	232.6 c
LRS 01/11	5.9 c	7.1 c	236.7 c
LRS 69/11	6.4 c	8.1 c	231.1 c
LRS 06/12	6.8 c	7.5 c	247.1 c
LRS 14/11	6.8 c	10.2 c	270.7 c
LRS 18/11	6.8 c	10.5 c	280.6 c
Mean (isolates)	5.0	6.4	201.0
CV(%)	24.8	25.9	25.5

[1]AUELPC was obtained from 13 evaluations at intervals of 3-4 days, for 50 days. [2]Means followed by the same letter in the column do not differ by Tukey's test at 5% probability. Statistical analysis with the data transformed into √x.

Most of the reported stem or collar inoculation methods with *P. cinnamomi* require previous wounding of the host tissues. They include drilling into host tissue and inserting plugs of mycelial agar (TIPPETT et al. 1983), incising the bark and inoculation with colonized Miracloth discs (PILBEAM et al. 2000) or colonized mycelial plug (BUNNY et al. 1995; NAIDOO et al.; 2011; RODRIGUEZ, 2016). In *Eucalyptus marginata*, as in the present study, all plants were infected when previously wounded but not all were infected when unwounded (O'GARA et al., 1996). These results, whilst supporting the widely accepted view that wounds are an important avenue for host tissue invasion by the pathogen, they also demonstrated that wounds are not essential for successful invasion through tissue. Regarding the progress of the invasion of avocado woody stems by *P. cinnamomi*, the inner bark and outer layer of wood are invaded, killing the phloem and xylem. On cutting through the bark a distinct reddish brown to brown discoloration of the underlying wood is evident (DANN et al., 2013). This can explain the greater internal size of lesions in relation to the external lesions in the inoculated seedlings in the present study (Table 1). In some hosts, as *E. marginata,* the lesions are not visible from external examination of the stems; however, after removal of the periderm layer, dark lesions are evident against the vivid green of the healthy phloem (O'GARA et al., 1996).

Considering inoculation without previous wounding, the mean length of external and internal lesions was 6.0 and 7.2 cm, respectively, for symptomatic plants (data not shown), close to that observed in plants inoculated with wound (Table 1). RODRIGUEZ (2016) reported that differences in the diseased area in the collar region of avocado seedlings 'Toro Canyon' and 'Duke 7' were not observed in plants inoculated with or without previous wounding, using similar inoculation methodology, supporting the idea that after infection, the development of the disease is similar, regardless of the penetration of the pathogen having been favored by wounding. Symptoms of leaf wilting were observed from six days after inoculation, and plant death from 15 days of inoculation. Four plants died as a result of the disease in inoculations without previous wounding while three plants died in inoculations with previous wounding, and the number of dead plants per isolate was not greater than one. Reisolation of the pathogen from the lesions in AW medium was possible for all 12 inoculated isolates, confirming their pathogenicity to avocado plants.

As in the present study, Belisle et al. (2019) also identified significant differences in virulence within California, USA, *P. cinnamomi* isolates from avocado. Evaluating the pathogen inoculation methodology in the collar of avocado seedlings, Rodriguez (2016) also found differences in the aggressiveness of two pathogenic isolates. Despite the occurrence of differences between isolates in terms of aggressiveness, isozyme, microsatellites and mitochondrial haplotypes studies on *P cinnamomi* populations from different regions of the world have revealed that, in general, genetic and genotypic diversity among isolates is low in most regions (OLD et al., 1984, 1988; LINDE et al., 1997; MARTIN; COFEY, 2012; ENGELBRECHT et al., 2017). In particular, in Australasian and South African populations, isozyme and microsatellites analysis indicated that low levels of space-temporal genetic diversity, higher frequency of A2 mating type and the absence of sexual reproduction, are common features among *P. cinnamomi* isolates (OLD et al., 1984, 1988; LINDE et al., 1997, ENGELBRECHT et al., 2017).

Effect *Phytophthora cinnamomi* inoculum concentration x flooding

'Hass' avocado seedlings inoculated with 1.0% (m/v) pathogen concentration and not subjected to flooding showed greater visual severity (%) of diseased roots than non-inoculated plants (Table 3). In the presence of the pathogen and regardless of the flooding period, there were no significant differences between the inoculum concentrations for the evaluated plant parameters, except LAI in 'Hass' plants subjected to 12h flooding (Table 2), possibly because *P. cinnamomi* has a short generation time and a high reproductive capacity. Inoculum can increase from low, often undetectable levels, to high levels within a few days, particularly when soils are warm, moist and well aerated, and food bases (feeder roots) are in abundance. The process of zoospore production can occur in less than 48 h and hence the pathogen has the capacity to produce millions of spores in a short period (DANN et al., 2013).

Table 2. Effect of flooding period and inoculum concentration of *Phytophthora cinnamomi* on the number of leaves, collar diameter, plant height and leaf area index (LAI) of 'Margarida' and 'Hass' avocado plants after two months of inoculation with the pathogen.

Flooding period (hours)	'Margarida' avocado plants			'Hass' avocado plants		
	Inoculum concentration (%)					
	0	0.1	1	0	0.1	1
	Number of leaves (Difference from before inoculation)					
0	24.8 (15.4 aA)	21.6 (12.6 aB)	28.2 (18.0 aB)	12.8 (4.0 aA)	11.0 (3.6 aB)	11.6 (3.8 aA[1])
12	29.2 (18.6 bA)	14.8 (4.8 aA)	17.6 (8.2 aA)	9.2 (0.8 aA)	6.0 (-2.0 aA)	8.0 (-0.6 aA)
24	23.0 (12.2 bA)	16.0 (5.8 aA)	15.0 (4.8 aA)	9.8 (1.8 aA)	7.4 (-0.2 aAB)	9.6 (1.6 aA)
CV(%)		20.75			28,16	
	Collar diameter (mm) (Difference from before inoculation)					
0	10.4 (4.5 aA)	9.2 (3.6 aB)	10.8 (4.6 aB)	6.5 (1.3 aA)	6.2 (1.3 aA)	6.4 (1.6 aA)
12	10.2 (4.1 bA)	8.5 (2.2 aA)	9.5 (3.1 abAB)	5.7 (0.8 aA)	5.5 (0.7 aA)	5.6 (0.8 aA)
24	9.6 (3.4 aA)	8.0 (2.4 aAB)	8.8 (2.4 aA)	6.2 (1.4 bA)	5.1 (0.5 aA)	5.5 (0.7 abA)
CV(%)		12.45			20,18	
	Plant height (cm) (Difference from before inoculation)					
0	62.6 (32.7 aA)	56.3 (28.3 aB)	64.1 (30.6 aB)	35.9 (8.3 aA)	37.3 (9.7 aB)	39.7 (11.4 aB)
12	50.3 (22.8 aA)	44.9 (12.4 aA)	52.6 (18.2 aAB)	32.8 (5.0 aA)	28.1 (0.4 aA)	31.8 (3.2 aA)
24	53.3 (23.9 bA)	37.4 (10.9 aA)	42.8 (11.9 aA)	37.2 (7.4 bA)	30.4 (0.3 aA)	30.8 (2.2 abA)
CV(%)		21.93			33,21	
	LAI (Difference from before inoculation)					
0	1.7 (1.34 aB)	1.6 (1.12 aA)	1.6 (1.17 aA)	0.7 (0.29 aA)	0.5 (0.12 aB)	0.8 (0.45 aA)
12	1.1 (0.50 aA)	0.9 (0.55 aA)	1.2 (0.74 aA)	0.3 (0.01 abA)	0.2 (-0.25 aA)	0.5 (0.16 bA)
24	1.1 (0.72 aAB)	1.0 (0.56 aA)	1.2 (0.61 aA)	0.5 (0.08 aA)	0.2 (-0.11aAB)	0.5 (0.16 aA)
CV(%)		24.58			17,21	

[1]Data for each cultivar followed by the same letter, lowercase on the line and uppercase on the column, do not differ by Tukey's test at 5% probability. Statistical analysis with the data transformed into $\sqrt{x + 0.5}$.

Table 3. Effect of flooding period and *Phytophthora cinnamomi* inoculum concentration on the dry mass of 'Margarida' and 'Hass' avocado plants and percentage of diseased roots.

Flooding period (hours)	'Margarida' avocado plants			'Hass' avocado plants		
	Inoculum concentration (%)					
	0	0.1	1	0	0.1	1
	Dry mass (g) of the aerial part					
0	25.4 aA	19.6 aB	24.9 aB	6.1 aA	5.1 aA	6.5 aA[1]
12	20.4 aA	11.4 bAB	16.2 abAB	3.3 aA	2.8 aA	3.9 aA
24	16.2 aA	9.8 aA	13.2 aA	4.9 aA	2.9 aA	3.9 aA
CV(%)		17.2			27,4	
	Dry mass (g) of the roots					
0	10.6 aA	7.0 aB	9.7 aB	3.1 aA	3.2 aB	3.8 aB
12	6.6 aA	2.7 bA	3.7 abA	2.1 aA	1.4 aAB	1.2 aA
24	6.9 aA	2.8 bA	3.5 bA	2.4 aA	1.0 aA	1.4 aA
CV(%)		22.7			28.1	
	Percentage of mass of diseased roots					
0	1.6 aA	12.9 aA	1.3 aA	0.4 aA	1.2 aA	4.9 aA
12	33.1 aB	67.0 bB	60.7 abB	11.9 aA	49.4 bB	47.3 bB
24	14.9 aAB	71.4 bB	72.0 bB	4.7 aA	65.3 bB	62.9 bB
CV(%)		30.6			22,1	
	Visual severity (%)					
0	1.2 aA	14.5 aA	2.4 aA	0.6 aA	1.8 abA	8.8 bA
12	47.7 aB	74.8 aB	79.4 aB	12.7 aB	60.6 bB	70.0 bB
24	15.9 aA	88.0 bB	85.0 bB	3.8 aAB	76.6 bB	77.0 bB
CV(%)		31.9			25.1	

[1]Data for each cultivar followed by the same letter, lowercase on the line and uppercase on the column, do not differ by Tukey's test at 5% probability. Statistical analysis with the data transformed into $\sqrt{x}$.

The interaction between flooding period and inoculum concentration was significant for the number of leaves in 'Margarida' plants ($p = 0.023$), which showed reduced number of leaves due to flooding associated with the presence of the pathogen, but such a reduction was not observed in the absence of pathogen or flooding (Table 2). A significant interaction ($p < 0.05$) was also observed for both studied avocado cultivars considering the percentage of diseased root mass and visual severity of diseased roots (Table 3). For the cultivar 'Margarida', there was no significant increase in the percentage of diseased roots in the absence of flooding, regardless of the inoculum concentration; however, 24 h flooding led to a higher percentage of diseased roots in the presence of the pathogen, while with 12 h flooding a significant increase in the disease was observed only in the proportion of diseased root mass at 0.1% of inoculum concentration (Table 3). 'Hass' plants had higher percentages of diseased roots with the association of flooding and pathogen inoculum, showing no differences in the percentage of diseased root mass in the absence of flooding or in the absence of the pathogen (Table 3). In general, regardless of occurrence of interaction between flooding period and inoculum concentration, these two stressing factors compromised the development of 'Hass' and 'Margarida' avocado seedlings (Tables 2 and 3).

'Margarida' avocado seedlings not inoculated with *P. cinnamomi* had their development negatively affected by 12 h and/or 24 h substrate flooding and showed significant differences from the treatment without flooding for leaf area index (LAI) and percentage of diseased roots, considering both the proportion of root mass and the visual severity (Tables 2 and 3). For the cultivar 'Hass', greater visual severity of diseased roots due to flooding was also found (Table 3). Root rot in plants not inoculated by the pathogen is most likely due to the partial anoxia of roots during substrate flooding periods, since avocado feeder roots are extremely sensitive to anaerobic conditions (SCHAFFER et al., 2013). Waterlogged conditions decreased biomass production with smaller leaf area (DOUPIS et al., 2017). According to BESOAIN et al. (2005), in 'Mexicola' avocado seedlings subjected to six cycles at fortnightly intervals of 0, 12, 24, 48 and 96 hours of flooding, the percentage of diseased roots reached 11.3% root mass after 48 h flooding. In the present study, with 12 h flooding such percentage reached 11.9% in 'Hass' (Table 3) and 33.1% in 'Margarida' (Table 3) avocado seedlings.

In Colombia, oxygen deficit in the soil is reported as one of the most frequent causes of death of avocado trees in the field, as a direct consequence of planting in flooded soils (GIL et al., 2014). Waterlogged or flooded soils may result from high rainfall, river overflow, elevated water tables, inadequate drainage and improper irrigation management (PANDEY et al., 2010). Avocado is a flood-sensitive species and flooding exacerbates the effect of PRR (REEKSTING et al., 2014).

According to Bensoain et al. (2005), the percentage of root rot reached 5.5% in 'Mexicola' seedlings not inoculated with the pathogen and subjected to six fortnightly flooding periods of, on average, 0, 12, 24 and 48 hours. In the present study, root rot increased with the inoculation of the pathogen, reaching 43.7% of the root system. 'Mexicola' avocado seedlings inoculated with the pathogen and exposed to six cycles of 96h flooding showed 100% diseased roots (BENSOAIN et al., 2005). The association of flooding and *P. cinnamomi* also had a positive synergistic effect in reducing the growth of the avocado rootstock in Kenya, resulting in reduced stem diameter, leaf area, plant heights, plant fresh weights and plant dry weights (SHIRANDA, 2018). Root infection by *P. cinnamomi* increases in the presence of free water, and a synergism exists between flooding and PRR outbreaks (PLOETZ; SCHAFFER, 1989; FARROW et al., 2011). The presence of excess water in soil initiates sporangia production and facilitates free movement of released zoospores towards roots, leading to high rates of infection (NIELSEN, 2016). Short periods of soil saturation with aerated water favors *P. cinnamomi*, whereas prolonged periods of soil saturation lead to anoxia which will damage or kill avocado roots and inhibit the pathogen (NIELSEN, 2016).

The first wilting symptoms were noticed from 26 days after inoculation in 'Hass' and from 32 days after inoculation in 'Margarida' plants. At the end of the experiment, symptoms were detected in 26.7% of 'Margarida' and 31.1% of 'Hass' plants, of which, one (2.2%) and three (6.7%) were dead, respectively. In the 'Hass' cultivar, wilting did not occur in non-inoculated plants but was detected in 46.7% of inoculated plants; however, in the absence of flooding, only 20% of inoculated plants showed wilting. In the 'Margarida' cultivar, wilting symptoms only occurred in plants subjected to a certain flooding period, which accounted for 40% plants, while only 10% plants subjected to flooding had wilting in the absence of the pathogen, evidencing that wilting symptoms may occur due to the presence of the pathogen or the occurrence of flooding, and that symptoms are intensified when both factors are associated.

Besoain et al. (2005) reported that, regardless of the soil flooding period, inoculated 'Mexicola' avocado plants presented wilting, reduced growth, smaller number of leaves and reduced leaf surface, which are symptoms always associated with the presence of partial or total rot of absorbing roots, eventually determining the death of plants. However, avocado trees can often tolerate a degree of root rot with no obvious effects on above-ground tree health (PLOETZ; PARRADO, 1988). Reduced photosynthesis, transpiration and stomatal conductance can also be detected in root rot-affected trees before visible

symptoms of disease become evident (WHILEY et al., 1986; PLOETZ; SCHAFFER, 1989).

P. cinnamomi was recovered from 63% root fragments from inoculated 'Hass' and 'Margarida' plants, via isolation in selective culture medium, showing no differences in the cultivars as a function of inoculum concentration and flooding periods. The pathogen could not be isolated from non-inoculated plants, evidencing the original health of seedlings and the absence of subsequent contamination among plants of the remaining treatments.

During the two months of experiment, minimum, mean and maximum temperatures inside the greenhouse were 12.8, 21.3 and 29.9 °C, respectively, within the adequate range for the occurrence of the disease. Disease develops optimally at temperatures between 19-25 ^{O}C and declines at > 30 ^{O}C and < 12 ^{O}C. Root rot is most severe at lower temperatures, where *P. cinnamomi* grows better than avocado trees, and is much reduced at higher temperatures that favor the host (DANN et al., 2013). According to Piccinin et al. (2016), the disease is completely inhibited at temperatures above 33 °C.

Combined stresses can act synergistically to reduce crop production such as root rot, poor aeration, and waterlogging (RAMÍREZ-GIL et al., 2020). Knowledge pertaining to the physiological and growth tolerance of avocado rootstocks to *P. cinnamomi* and flooding is limited. Differences in tolerance to flooding were already observed in several avocado rootstocks, although, in general, they were not related to tolerance to *P. cinnamomi*. The South African rootstock selection R0.06 exhibits superior tolerance to flooding and PRR, but not when these two stresses are combined (REEKSTING et al., 2014). Therefore, rootstock selection should consider adaptation to several combined stresses which suggests that local edaphoclimatic conditions require specific tests of rootstock selection. In a recent evaluation of eleven native avocado genotypes in Colombia, different levels of adaptability to drought and flooding conditions were observed, as well as some degree of resistance to PRR. In general, West Indian-type genotypes showed better adaptability to higher soil moisture conditions and showed lower values of PRR, while Guatemalan-type genotypes showed advantages for drought conditions and intermediate tolerance to PRR and hypoxia/anoxia (RAMÍREZ-GIL et al., 2020). In another study with 'Fuerte', 'Puebla', 'Pinkerton' and 'Booth7' rootstocks, 'Fuerte' and 'Puebla' showed greater tolerance to *P. cinnamomi* and flooding treatments (SHIRANDA, 2018).

Conclusions

The avocado *P. cinnamomi* isolates showed virulence variability and the lesion length produced by the pathogen in the collar region of inoculated avocado seedlings previously wounded proved to be a simple and effective technique for assessing t the aggressiveness of *P. cinnamomi* isolates.

P. cinnamomi at concentration of 0.1 – 1.0% (m/v) in the substrate and fortnightly 12 - 24 h substrate flooding compromised the health of the root system of avocado seedlings. However, combination of the two stresses brings about an additive effect to the disease symptoms. Thus, in pathogenicity and disease control studies using soilless potting media, flooding period after inoculation is recommended for obtaining high rates of *P. cinnamomi* infection.

Despite the limitations to extrapolate these results to commercial conditions, avoiding waterlogged conditions in avocado orchards is therefore of fundamental importance in maintaining healthy and productive trees.

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