Investigation note

Dynamics of *in vitro* growth of *Phytophthora cinnamomic* in alternative culture media

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Abstract

Mexico is the leading producer, consumer and exporter of avocado in the world. The study of soil macrobiotics becomes more important in the avocado wilt complex because in these pathology biotic and abiotic causative agents can be implicated, being the Oomycete *Phytophthora cinnamomi* Rands the main radical disease of avocado cultivation. The objective of the present investigation was to evaluate the dynamics of the *in vitro* growth of *Phytophthora cinnamomi* in alternative culture media. In the months of October and November 2016, root samples were collected from avocado trees (*Persea americana* Mill. Var. Hass) in the INIFAP experimental orchard located in San Juan Nuevo Parangaricutiro, Michoacán. The isolates were identified morphologically and molecularly. The measurements of the growth diameters of each strain were recorded in vitro according to time. Linear regression was carried out using generalized linear models (GLM) to obtain the parameters of intercept and slope, significance of the regression and the R^2 value adjusted. The analyzes were carried out using the statistical program R 3.4. According to the results obtained, the culture medium Rye-agar was the most efficient for the proliferation of *P. cinnamomi*.

Keywords: avocado, culture media, growth rate.

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P. americana Mill.belongs to the *Lauraceae* family, one of the oldest angiosperm groups (Renner, 1999). It is characterized by its great variability and for being one of the most numerous families of the plant kingdom, with around 92 genera described and an indeterminate number of species that varies from 2 840 to 3 340 that are distributed throughout the tropical and subtropical regions of the world (Renner, 2004). The productivity of the crop in this wide range of environments depends on a set of factors, some more or less linked to the agri-environmental characteristics of the orchards.

However, biotic and abiotic factors are limiting to obtain good yields and expected fruit quality (Anguiano *et al*., 2003). Soil microbiota can serve as a bio-indicator of the health of ecosystems and agro-ecosystems, reflecting the effect of agricultural processes and the management of resources on this component, affecting both their biodiversity and the density of populations microbials (Buckley and Schmidt, 2003; Girvan *et al*., 2004; Ariena *et al.*, 2006).

The study of soil macrobiotics becomes more important in the avocado wilt complex because in these pathology biotic and abiotic causal agents may be implicated, being the Oomycete *Phytophthora cinnamomi*, the most relevant, which can cause considerable economic losses (Pérez, 2008; Richter *et al*., 2011). The disease was described in 1922 by Rands in cinnamon trees, it is reported attacking avocado for the first time in the US in California, it is considered the most important disease of the avocado, for the decade of the 1980's had 1 500 ha been affected (Zentmyer, 1980).

In Mexico, the presence of the disease known as avocado sadness has been detected in all the producing areas; emphasizing by the severity of the damages, the region of Atlixco, Puebla, where it caused the disappearance of this crop. In the production region of Michoacán, it is considered that around 4 000 ha are affected by the disease, presenting an exponential trend (Telisz, 2000).

In this sense, it is necessary to have extensive knowledge of the *in vitro* behavior of *P. cinnamomi* and for this, it is necessary to start from alternative culture media to accelerate its development and therefore make rapid assessments regarding its growth since this phytopathogen is affected in its *in vitro* development by different factors such as temperature and one option is to generate information from culture media that accelerate its growth rate.

It should be noted that to date there is limited information on alternative growing media to the PDA and V8 Agar. Therefore, the objective of the present investigation was to evaluate the dynamics of *in vitro* growth of *P. cinnamomi* in four alternative culture media.

Materials and methods

Sampling

In the months of October and November 2016, root samples were collected in avocado trees (*Persea americana* Mill. var. Hass) under inoculum pressure that presented characteristic symptomatology of descending death known as avocado sadness. The collection site was the experimental garden of the National Institute of Forestry, Agriculture and Livestock Research (INIFAP) located in San Juan Nuevo Parangaricutiro, Michoacán, whose semi-warm, sub-humid

climatic conditions with rain in summer, range from 1 200 to 1 600 mm and temperatures of 10 to 28 °C (García, 1981). A directed sampling was carried out close to the drip area at a depth of 30 cm at four equidistant points.

With the help of a straight shovel, the root samples were taken with the presence of damage (necrotic dark brown tissue) of 2 to 6 mm in diameter and placed in polyethylene bags previously labeled with the data from the garden, municipality, georeferencing and agronomic management (conventional or organic), later they were transported to the phytopathology laboratory of the Department of Agricultural Parasitology at the Agrarian Autonomous University Antonio Narro.

Isolates of phytopathogens

The roots were washed with sterile distilled water to fragment them into pieces no larger than 0.5 cm, with the support of a sterile scalpel, a longitudinal cut was made selecting the limits of healthy and diseased tissue, the root cuts were washed in 3% sodium hypochlorite solution for one min, followed by three washes with sterile distilled water and placed on absorbent paper previously sterilized. Once dried, V8®-PARPH was planted in a selective medium placing four pieces of roots horizontally in 8.5 cm diameter Petri dishes; three boxes per sample, giving a total of 12 roots per tree sampled and finally the isolates were incubated at 28 °C for three days in total darkness (Fierro, 2011).

Purification and multiplication

V8®-Agar strains with characteristic growth of *P. cinnamomi* were transferred to culture medium. The purification technique used was by tip of hypha in triplicate, placed in the center of the Petri dish, sealed with cling wrap and incubated at 28 °C in a bioclimatic chamber for 72 h in the Phytopathology laboratory of the Department of Agricultural Parasitology.

Morphological and molecular identification

The pathogens obtained were identified morphologically according to Davison and Ribeiro (1996) and molecularly through the PCR-ITS technique, extracting DNA according to the methodology of Doyle and Doyle (1990); from 0.2 g of mycelium from the pure culture with lysis buffer (50 mM EDTA, pH 8.5, 100 mM Tris HCl, pH 8, 50 mM NaCl, 2% SDS). DNA visualization was performed on a 2% agarose gel stained with GelRed (GenScript®).

The amplification of the ITS region was carried out with the primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'- TCCTCCGCTTATTGATATGC-3'). In the same way, the product of the reaction was visualized by means of electrophoresis in 2% agarose gel stained with GelRed (GenScript®) and the PCR product was sent to the phytosanitary diagnostic laboratory UA-LAB.

Evaluation of the growth rate

The culture media were potato-dextrose-agar (PDA), Pea-Agar, Rye-Agar and V8®-Agar. Three strains with different characteristic growth (cameloid, rosette and stellate) were selected, later for each medium, they were sown in the center of a Petri dish of 8.5 cm in diameter 5 mm diameter

explants taken from the margin of a vigorous growth of a colony 8 days after sowing with four repetitions, the boxes were sealed and incubated in bioclimatic chamber where they grew as pure isolation at 28 ±2 °C in total darkness, with the help of a digital vernier, diameter readings were taken they were taken every 24 h at four equidistant points until the last repetition filled the box.

Statistical analysis: once having the measurements of the diameters of each strain in different nutrient medium, linear regression was performed by generalized linear models (GLM) to obtain the parameters of intercept and slope (growth rate), significance of the regression and the value of R^2 adjusted. The homogeneity of variance test of the errors of the regression models in each species was carried out and the linear coefficients were contrasted with time, using the values of the sum of squares of each regression by means of a test of t (Ott and Longnecker , 2010) using the following expressions:

$$t = \frac{b_1 - b_2}{\sqrt{Sp\left[\frac{1}{ss_1}\right] + \left[\frac{1}{ss_2}\right]}} \quad (1)$$

Where: b_1 and b_2 are the slopes of each regression for each isolation; SS_1 and SS_2 are the sum of squares of each regression model of the equations.

$$Sp = \frac{MSE_1 - MSE_2}{2} \quad (2)$$

Where: Sp= is the combined estimate of the error of the two regression equations with n-2 degrees of freedom. SME= mean square of the experimental error.

The analyzes were performed using the statistical program R 3.4®.

Results and discussion

Derived from the isolation and morphological and molecular identification of the phytopathogens obtained from the samples taken in the experimental garden under inoculum pressure, different strains of the same species were obtained, coinciding with the identification keys of Davison and Ribeiro (1996) for *P. cinnamomi*, confirming the above mentioned when comparing the sequencing products with the records of the GenBank database (Table 1).

Table 1. Molecular characterization of the isolates of the sequences reported in the gene bank with the intergenic sequences (ITS) of the rDNA genes.

Strain[1]	No. access[2]	Species	Similarity[3]	Origin[4]
Pc-33	LN846114.1	*Phytophthora cinnamomi*	99%	Canary Islands
Pc-41	LN846114.1	*Phytophthora cinnamomi*	99%	Canary Islands
Pc-42	KP183223.1	*Phytophthora cinnamomi*	99%	Australia B

[1]= nomenclature for different isolates; [2]= access number in the NCBI database (National Center of Biotechnology Information). [3]= similarity index between sequences of isolated species and compared species. [4]= geographical origin of the isolates.

Regarding the temporal evaluation of the growth of *P. cinnamomi* in different culture media (Figure 1), it was observed that the strains in the different media (Table 2), all tend to be adjusted to a linear growth curve, given that in the regressions adjusted R^2 are greater than 0.94, all highly significant (α= 0.05).

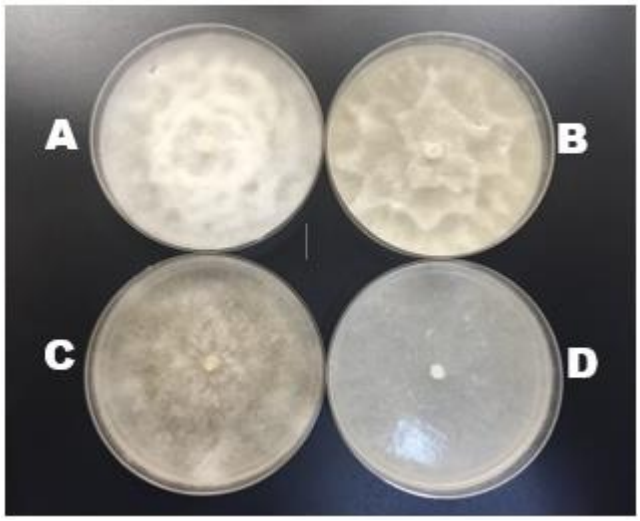

Figure 1. Growth morphology in the different culture media where A= PDA; B= Pea-Agar; C= V8®-Agar; and D= Rye-Agar.

Table 2. Growth rate in cm h of the evaluated media.

Medium	Treatment	Intercept	Parameter	*p*- value[b]	R^2 aj.
V8	Pc-33	0.952	0.041142	< 2.2e-16	0.985
	Pc-41	0.411	0.038884	< 2.2e-16	0.985
	Pc-42	0.642	0.050434	< 2.2e-16	0.958
Rye	Pc-33	0.731	0.067878	< 2.2e-16	0.976
	Pc-41	0.27	0.070667	< 2.2e-16	0.949
	Pc-42	0.585	0.06916	< 2.2e-16	0.978
Pea	Pc-33	1.02	0.067108	< 2.2e-16	0.959
	Pc-41	1.028	0.065788	< 2.2e-16	0.967
	Pc-42	1.052	0.068528	< 2.2e-16	0.944
PDA	Pc-33	0.04	0.039919	< 2.2e-16	0.967
	Pc-41	0.04	0.037524	< 2.2e-16	0.979
	Pc-42	0.0519	0.038151	< 2.2e-16	0.973

b= *p* values <0.05 are highly significant, aj.= adjusted.

The nutrient medium in which the lowest growth rates were recorded was PDA, with an average of 0.0385, with Pc-41 being the lowest with respect to the others, followed by the V8 medium with an average of 0.0434, on the other hand, greater values were presented in Rye and Pea with means of 0.0671 and 0.0692 respectively, being in the latter where the maximum value in the Pc-41 strain

was recorded being 0.03314 cm greater with a ratio of 1.8832, reports a growth of 4.5 cm in 120 h of the same pathogen, coinciding broadly with these results; however, estimating the growth of the strains evaluated in this study, the average speed was 4.5 cm in 85 h.

When comparing the strains with the different media, a statistical difference was determined between them, for the case of strain Pc-33, the differences are between the means Pea-PDA and Rye-V8, on the other hand when contrasting Pc-33 with Pc-41 significant means were PDA-Pea and PDA-Rye, same result comparing it with strain Pc-42, this given that PDA has the lowest rates (Table 3), contrary to that reported by Rodríguez *et al*. (2003) who recorded high growth values, taking into account the same mm d^{-1} measurement for the oomycete *P. cinnamomi*.

Table 3. Student t values resulting from multiple comparisons of the standardization of growth rates (cm h^{-1}) of different strains of *P. cinnamomi* in different nutrient media.

		Pc-33				Pc-41				Pc-42			
		M1	M2	M3	M4	M1	M2	M3	M4	M1	M2	M3	M4
Pc-33	M1	-											
	M2	0.044	-										
	M3	2.041	4.693*	-									
	M4	4.249*	1.803	-0.118	-								
Pc-41	M1	-0.102	-0.166	-1.621	-1.245	-							
	M2	0.133	0.084	-12.982**	-1.896	0.094	-						
	M3	4.74*	2.142	0.237	1.997	1.451	1.831	-					
	M4	4.696*	1.732	0.084	0.299	1.137	1.769	-0.184	-				
Pc-42	M1	-0.32	-0.116	-2.101	-3.87*	0.055	-0.207	-4.372*	-6.033*	-			
	M2	-0.026	-0.081	-1.79	-1.29	0.172	-0.143	-1.516	1.346	0.0258	-		
	M3	3.786*	2.24	0.19	0.682	1.461	2.414	-0.162	0.088	3.646*	1.533	-	
	M4	0.963	2.817	-1.157	-0.617	1.194	1.343	-0.9	-0.717	1.025	1.408	-0.899	-

Culture media: M1= Rye; M2= Pea; M3= PDA; M4= V8; **, *= statistically different values with an α= 0.05 and 0.1 respectively.

Regarding strain Pc-41, the growth rate is indistinct in the medium sown because there is no difference in function to the nutrient medium, however, in contrast to Pc-42, the growth is more accelerated in rye. It is important to note that, despite the aforementioned, the culture medium does influence the growth of reproductive structures. Finally, Pc-42 shows significance in the media rye and PDA being the value in Rye 1.8x larger with respect to PDA.

Based on the results of Table 2 and the comparisons in Table 3, the Rye and Pea media are viable and alternative means for the PDA medium widely used for its higher growth rate and Cedeño *et al.* (2008) suggests determining alternative means for each pathogen due to the highly significant growth ranges.

Conclusions

According to the results obtained, the Rye-agar culture medium was the most efficient for the isolation and proliferation of *Phytophthora cinnamomi* obtaining a greater growth of morphological structures. The information generated will help future investigations for the rapid isolation and formation of *Phytophthora cinnamomi* structures.

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