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Effectiveness of carboxylic acids from *Pichia membranifaciens* against coffee rust

Eficácia do ácido carboxílico de *Pichia membranifaciens* contra a ferrugem do cafeeiro

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ABSTRACT

Coffee rust is a fungal disease that has affected every coffee-producing region in the world. Given that the effectivity of the protectant and systemic fungicides applied routinely to control the spread of the causative agent of the disease (*Hemileia vastatrix*) has gradually diminished, besides are harmful to mammals and ecosystems, the objective of this work was to search for a mixture of harmless natural compounds with the potential to be applied in the field. So, a yeast strain producing a battery of long-chain carboxylic acids (CA) with fungicide properties was isolated from soil of coffee crop and identified as *Pichia membranifaciens* by ITS sequencing. Culture conditions of the yeast were optimized and the CA in the solution were characterized by Gas Chromatography-Mass Spectrometry (GC-MS) as ethyl formate (55.5 g L^{-1}), octadecenoic acid (3.5 g L^{-1}), propionic acid (7.2 g L^{-1}), 3-(octadecanoyl)-propionic acid (7.2 g L^{-1}) and methyl acetate (8.4 g L^{-1}). Randomized field studies were conducted in three different locations in Chiapas, México. Five treatments were tested including three concentrations of the CA solution (389, 584 and 778 ppm) and copper oxychloride (5 000 ppm) as conventional control. The initial coffee rust incidence averages varied between sites: Maravillas (3-9%), Santo Domingo (10-16%) and Búcaro (16-22%). The treatments of CA solution proved to be effective at slowing down the progress of the rust disease even for the sites where initial incidence was high. Likewise, the CA solution reduced the viability of *H. vastatrix* spores, as assessed by fluorescence microscopy.

Index terms: Natural compounds; yeast identification; field studies; antagonism.

RESUMO

A ferrugem do cafeeiro é uma doença que tem afetado as regiões produtoras de café no mundo. Considerando que os funguicidas protetores e sistemáticos que são aplicados rotinariamente para controlar a difusão do agente causador da doença (*Hemileia vastatrix*) são nocivos para os humanos, animais e ecossistemas, o objetivo deste trabalho é determinar um componente natural com potencial de uso. Uma cepa de levedura produzindo uma bateria de ácidos carboxílicos com ação funguicida foi isolada do solo e identificada como *Pichia membranifaciens* por sequenciamento ITS. Foram optimizadas as condições de cultura e os ácidos carboxílicos presentes na semi purificada sobrenadante (solução CA) foram caracterizados por cromatografia de gás espectometia de massa (GC-MS) como formato de etilo (55.5 g L^{-1}), ácido octadecenóico (3.5 g L^{-1}), ácido propiônico (7.2 g L^{-1}), 3-(octadecanoil)-ácido propiônico 7.2 g L^{-1}) e acetato de metilio (8.4 g L^{-1}). Estudos do campo foram conduzidos em três locais na região de Chiapas, Mexico. Cinco tratamentos foram testados incluindo três concentrações da solução CA (389, 584 e 778 ppm) e o funguicida comercial contendo oxicloreto de cobre (500 ppm). As médias iniciais de incidência da ferrugem do cafeeiro variou em função do local: Maravilla (3-9%), Santo domingo (10-16%) e Búcaro (16-22%). A solução CA provou ser efetiva retardando o progresso da doença, mesmo nos locais onde a incidência inicial foi elevada. A solução CA reduziu a viabilidade dos esporos de *H. Vastatrix,* observado por meio de fluorescência.

Termos para indexação: Compostos naturais; identificação de leveduras; estudos do campo; antagonismo.

INTRODUCTION

Coffee is one of the most valuable agricultural commodities in the world market (ICO, 2014). Latin America is the world's largest producing region, contributing more than 56% of the coffee world production (FAO, 2016). However, coffee rust, a fungal leaf disease caused by *Hemileia vastatrix,* has caused great economic losses since it was first detected in Brazil in 1970 and spread across the continent (Waller, 1982). Although the impact of the disease in each country within the region differs (Avelino et al., 2015), the 2012/13 season presented the worst epidemic ever recorded, with more than 50% of all coffee producing areas affected in Central America and Mexico (ICO, 2014).

Different strategies have been implemented in order to address the problem. In Colombia and Brazil a great number of susceptible Arabica coffee plantations were replanted with resistant varieties (Avelino et al., 2015; Zambolim, 2016). In other countries this strategy is not yet economically feasible (Avelino et al., 2015); in addition, resistance has not proved to be long lasting since *H. vastatrix* presents a great deal of genetic variability (Cristancho et al., 2014). Gouveia et al., 2005 described that considerable variations within South America populations suggest a high evolutionary rate of *H. vastatrix*. Recently, Nunes et. al, (2009) also reported high degree of genetic variation among *H. vastatrix* in Brazil.

Protectant and systemic fungicides have been used against *H. vastatrix*, either by themselves or in combined regimes (Mccook; Vandermeer, 2015). Although systemic fungicides have been effective (De Souza et al., 2011), best results are achieved when they are applied preventively. Nevertheless, this practice increases production costs, as well as environmental and human exposure (Hester et al., 2012).

Among protectant fungicides, copper based formulations were the most common for many decades (Waller, 1982) however, due to their increasing cost cost-effective systemic fungicides are nowadays becoming more successful (Zambolim, 2016). The main disadvantage of the use of any chemical compounds is that their accumulation in water and soil results in the alteration of the associated ecosystems (Giller et al., 1998). Thus, it is becoming increasingly necessary to implement rational and optimized control of this disease, due to low coffee prices and pollution problems.

Biocontrol products against coffee rust have been tested in order to lessen the environmental impact and health hazards of traditional fungicides (Georgopoulos et al., 2001; Hester et al., 2012). Bacterial and fungal antagonists as well as their derivative metabolic products have been tested *in vitro* and in field studies against *H. vastatrix* with positive outcomes (Carrinl; Rico-Gray, 2002; Haddad et al., 2014; Jackson et al., 2012). Although it has been stablished that CA naturally insert themselves into the lipid bi-layer of the fungal membranes and physically disturb the membrane (Pohl et al., 2011), these have yet to be tested as fungicides in the field (Liu et al., 2008), because in general, they were previously shown to only act at high concentrations (Hassan et al., 2015). Nevertheless, it has been demonstrated that combinations of specific CA can synergize to present a potent antifungal activity, even at low concentrations (Coleman et al., 2010; Hassan et al., 2015; Hsiao and Siebert, 1999).

In this study, we describe a strain of the yeast *Pichia membranifaciens* that is capable of producing a battery of CA with antifungal properties. *H. vastratix* spore-viability after exposure was assessed *in vitro* and the mixture of compounds produced by this yeast were tested in the field, where they showed a capacity to control the incidence of coffee rust that was better than some most used agricultural copper formulation in the regions. Hence, we conclude that the CA produced by *P. membranifaciens* have the potential to be used against coffee rust.

MATERIAL AND METHODS

Soil samples were collected from commercial coffee crops in México. All samples were kept at 10-15 °C during transportation to the laboratory, where they were suspended in distilled water. For yeast isolation, the aqueous phase was recovered by filtration, and serial dilutions were plated on Potato Dextrose Agar (PDA) and Sabouraud agar (Sigma-Aldrich, St. Louis, MO, USA). Pure cultures were characterized morphologically and by microscopy. *In vitro* tests were carried out to determine the fungicidal activity of each of the isolates against *Fusarium* spp and *Verticillium* spp using PDA solid media (Csutak et al., 2013).

A yeast strain exhibiting antifungal activity was selected and identified by ITS gene sequencing (Schoch et al., 2012). DNA extraction and PCR amplification were carried out as previously described (Edwards et al., 1991) using ITS1-F_KYO2 and ITS4 primers (Toju et al., 2012). PCR products were purified using an Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, IL, USA) and sequenced by Capillary electrophoresis (Genomic Services Unit, Langebio-CINVESTAV, Irapuato, México). The resulting sequences were analyzed using the Applied Biosystems Sequence Scanner V1.0 software (ThermoFisher Scientific Inc., Waltham, MA USA) and compared against sequences in NCBI database. In this way the strain was identified as *Pichia membranifaciens* KP860059.1.

Pichia membranifaciens cultures were optimized and escalated. The complex media used was based on sugar cane molasses (20 g L^{-1}) that were obtained from an industrial sugar manufacturing plant (Los Canastos, Durango, México). Inocula were grown overnight in 2 L of the same media at 30 °C and constant agitation (150 rpm). Polyethylene tanks (20 L) containing the previously described media were inoculated with 1 L of the inoculum (4.61×10^4 CFU mL^{-1}). The cultures were maintained at room temperature for 15 days with 2-hour agitation intervals every 24 hours. After centrifugation (3000 rpm), a Carboxylic Acid solution (CAs) was obtained, maintained at 70 °C for 15 min and clarified. Physical and physicochemical

properties were determined. Additionally, microbiological determinations were performed (Rompré et al., 2002). The Colony Forming Unit (CFU) was obtained after count the number of colonies on an inch^2 of a plate of Nutritive agar with the CAs supended preincuted 48 h at 37 °C.

The CAs (5 mL) was extracted with ethyl acetate, the organic phase was evaporated under reduced pressure and the residue was dissolved in 10 mL of isooctane (Sigma-Aldrich, St. Louis, MO, USA).

The resulting solution was analyzed by Gas Chromatography-Mass Spectrometry (GC-MS). The analysis was performed in an Agilent 6890 equipment (Agilent Technologies Inc., Wilmington, DE, USA) coupled to an ion trap detector (ITD Finnigan 700; ThermoFisher Scientific, Waltham, MA, USA) using an RSL-150 column (25 cm × 0.32 mm, 0.30 μm film thickness). The temperature settings for the injector and the detector were 250 and 240 °C, respectively.

The effect of the CAs on *H. vastatrix* was determined using a LIVE/DEAD BacLight bacterial viability kit (Molecular Probes, Inc. Eugene, OR, USA) (Hu; Murata; Zhang, 2017). Spores were obtained from a commercial plot in Chiapas, México and suspended in 1500 µL of phosphate-buffered saline buffer (PBS 1X pH 7.4). From the initial spore suspension, 350 µL aliquots were taken. After centrifugation (2500 rpm) and further washing with PBS buffer, the spores were resuspended in 1 µL of 1:100 or 1:200 dilutions of the CAs and incubated at room temperature for 72 h. Samples of 100 µL of the suspension were washed with PBS, resuspended in 20 µL of the same buffer and stained using an equal volume of a 1:1 mix of SYTO 9- propidium iodide. The samples were incubated for 30 min at 4 °C in the dark. The stained spores were washed twice with PBS, and 15 µL of the suspension were placed on microscope slides and immediately observed using a 40X objective on a Leica DM 6000 (Leica Microsystems, Wetzlar, Germany) epifluorescence microscope (GFP 480/535 nm and DSR 535/610 nm filters).

H. vastatrix spores treated with solutions of glutaraldehyde (2.5% in PBS) and *p*-formaldehyde (4% in PBS), as well as untreated spores, were stained in the same way using LIVE/DEAD BacLight reagents, as positive and negative controls respectively. A minimum of 100 spores per treatment were analyzed using ImageJ 1.51j8 Software (Wayne Rasband, National Institutes of Health, USA).

Field studies were carried out in 2014 (April to June) in three commercial planting sites in Tapachula, Chiapas, México (Santo Domingo, Finca Maravillas, and Búcaro) all of them with a plantation density of around 1300 plants per hectare. All the three sites presented a certain degree of variation in altitude, climate, sun coverage and topography, representing different coffee growing conditions as well as all sites grow Arabica Coffee plants (*Coffea arabica* cv *Borbonica*) under mixed shade and without the use of fungicidal compounds.

All sites have a tropical-wet (Am) climate, according to the Köppen-Geiger climate classification (Peel et al., 2007), annual precipitation and temperature averages vary within sites (Santo Domingo 4153 mm/21.4 °C; Maravillas 2653 mm/26.5 °C; Búcaro 2497 mm/27.5 °C) (Conagua, 2016). Also, given that identical shade conditions do not occur even within the same plantation, there were variations on the type and total shade coverage for the three sites. The five different treatments were tested in quadruplicate in each site. Experimental plots were distributed on the terrain in order to account for topography and shade variations on the location. Temperature and relative humidity were measured each day and expressed as the average per week (NOM-032-FITO-1995).

Four random plots were delimited in each site according to EPPO standards (Eppo, 2012). In each plot five treatments, including the CAs and a copper oxychloride agricultural formulation (OxiKing, Ducoragro S.A. de C.V., Jalisco, México), were tested.The treatments were divided in: (T1) No fungicidal compound applied (control); (T2) 1 L CAs in 200 L water (equivalent to 389 ppm); (T3) 1.5 L CAs in 200 L water (equivalent to 584 ppm); (T4) 2 L CAs in 200 L water (equivalent to 778 ppm) and (T5) 1 kg copper oxychloride in 200 L water (equivalent to 5 000 ppm).

In each random plot, 20 asymptomatic new leaves were selected from 10 plants each (Total=200 leaves) prior to floral bud development. Three applications were performed on the surface of the leaves in 14-day intervals. An 818.015 "Swissmex-Rapid" sprayer was used to deliver a spray density of 650 L/ha. Temperature and relative humidity were monitored *in situ* using a Hobo DataLogger equipment (OneTemp Try Ltd., Australia). The rainfall data of each month were obtained from a meteorological unit of the National Water Comision (CONAGUA) of Mexico (INEGI, 2015). Incidence (expressed as the percentage of coffee rust affected leaves from the total at each site) was obtained weekly after the first application. The severity of the disease was measured by the scale of the % of leaf area infection. No disease symptoms was counted as 0 while leaves with more than 50% affected area was counted as 5. In between, mild chlorotic spots was counted as 1, while 1-5%, 6-20% and 21-50% affected leaves was counted as 2, 3, and 4 respectively (Senasinca, 2016).

The results are expressed as the mean values with the standard error of mean (SEM). The collected data were statistically analyzed by employing analysis of variance (ANOVA) and treatment means were compared using Tukey test at 5% probability level (Steel; Torri, 1997). (Supplementary file 1)

RESULTS AND DISCUSSION

Pichia membranifaciens KP860059 reduced the growth of *Fusarium spp* and *Verticillium spp* expressed as their mycelial radius, by 81% and 71.3% compared to the control (Figure 1). As expected, *P. membranifaciens* has antifungal properties.

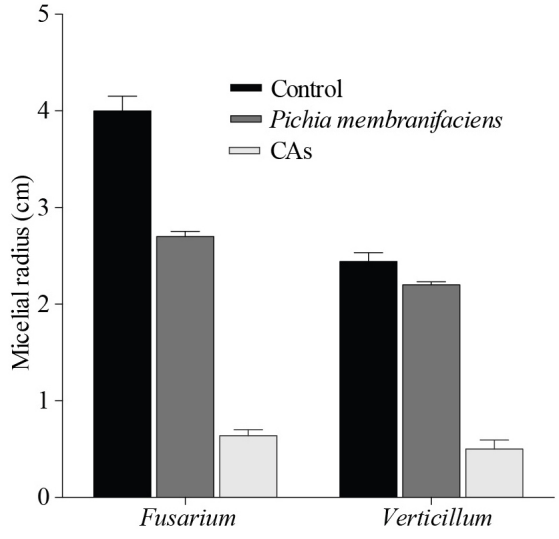


Figure 1: Effect of *Pichia membranifaciens* (KP860059.1) and CAs on the growth of *Fusarium* spp and *Verticillium* spp expressed as their mycelial radius.

Pichia membranifaciens is commonly found in soil samples and fruit surfaces. When growing on grape skins, it plays a role in wine spoilage by producing a series of metabolites that alter the flavor and aroma (Rankinei, 1964).

This species of yeast has been evaluated as a biocontrol agent against a variety of phytopathogenic fungi including *Penicillium expansum* (Cao et al., 2010), *Botrytis cinerea* (Lutz et al., 2013), *Rhizopus stolonifer* (Fan et al., 2000), *Monilinia fructicola* (Chan; Tian, 2005) and *Colletotrichum acutatum* (Cao et al., 2008). It's antagonism does not seem to be a consequence of a single mechanism of action; instead, volatile and diffusible compounds have been implicated as well as the expression of a killer toxin (Lutz et al., 2013).

The physical and physicochemical and microbiological properties of the current CAs is presented in Table 1. The color of CAs was found yellowish green with vinegar smell and with density of around 1.004 g/mL. The pH and conductivity were found to be 3.3 and 1485 µS/cm respectively. No trace of mesophiles or coliforms was found in the current CAs.

Table 1: Physical, physicochemical and microbiological properties of CAs produced by *Pichia membranifaciens*.

Properties	Value
Physical	
Color	Yellow green (99FFCC)
Smell	Vinegary, acid
Density	1.004 ± 0.039 g mL^{-1}
Physicochemical	
pH	3.320 ± 0.146
Conductivity	1485 µS/cm
Microbiological	
Mesophiles	0.00 CFU/mL
Total coliforms	0.00 CFU/mL

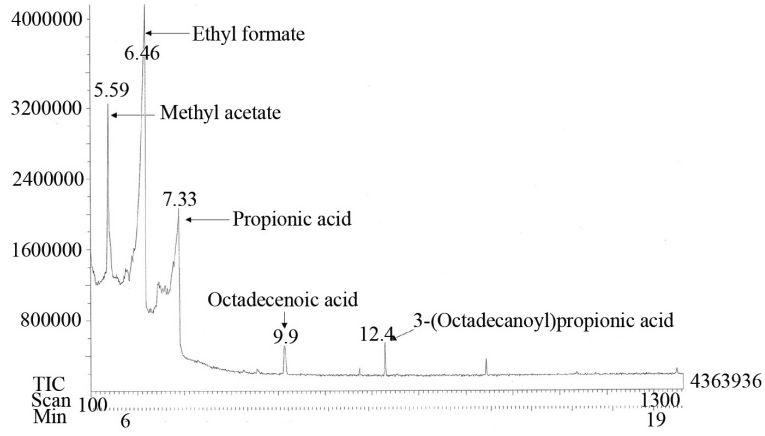
The GC-MS analysis of the compounds identified in the CAs were: ethyl formate (55.5 g L^{-1}), octadecenoic acid (3.50 g L^{-1}), propionic acid (7.20 g L^{-1}), 3-(octadecanoyl)-propionic acid (7.20 g L^{-1}) and methyl acetate (8.40 g L^{-1}) (Figure 2).

CA can display activity against bacteria, parasites and fungi (Hassan et al., 2015; Hsiao; Siebert, 1999). Although their main target seems to be the cell membrane, it is known that certain acids act upon a number of different pathways (Pohl et al., 2011), contributing to the synergistic antifungal effect observed for some carboxylic acid combinations (Coleman et al., 2010; Hassan et al., 2015).

A few CA, alone and in combination, have been tested as environment-friendly alternatives to control fungal crop diseases (Liu et al., 2008). In this work, we tested the particular combination of CA produced by *P. membranifaciens* KP860059.1 (CAs), grown in a complex carbohydrate media, against *H. vastatrix* fungi, the causative agent of Coffee rust.

LIVE/DEAD BacLight test differentiates between intact spores (green) and those with a compromised membrane (red). Due to their particular characteristics, a control of non-viable *H. vastatrix* urediniospores stained yellow, as seen in Figure 3, where glutaraldehyde and *p*-formaldehyde treated spores were used to fix the spores.

A



B

No.	Rt[a] (min)	Compound	Fragmentation pattern (*m/z*[b])
1	5.59	Methyl acetate	CH_3COOCH_3(74, 16.3%), $[CH_3COO]^+$ (60, 100.0%), $[CH_3CO]^+$ (43, 75.9%), $[CH_3]^+$(16, 10.6%)
2	6.45	Ethyl formate	CH_3CH_2COOH (74, 100.0%), $[CH_3COOH]^+$ (60, 45. 4%), $[CH_3CH_2O]^+$ (45, 62.1%), $[CHO]^+$ (29, 73.3%), $[CH_3CH_2]^+$(28, 87.6%)
3	7.35	Propionic acid	$[CH_3CH_2COO]^+$(73, 33.1%), $[CH_3COOH]^+$(60, 100.0%), $[CH_3CH_2]^+$(28, 23.8%)
4	9.92	Octadecenoic acid	$[C_{18}H_{33}O_2]^+$(281, 100.0%)
5	12.4	3-(Octadecanoyl)-propionic acid	$[C_{21}H_{39}O_4]^+$(355, 44.30%),$[C_{18}H_{35}O]^+$(267, 30.5%), $[C_3H_5O_2]^+$ (73, 100.0%)

Figure 2: GC-MS spectrum of CA (A) and fragmentation pattern (B), [a]Retention time (min), [b]mass/charge relation (*m/z*).

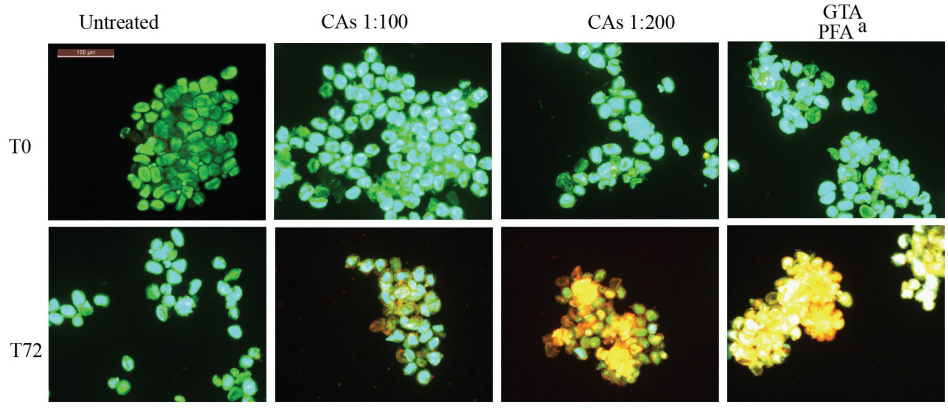


Figure 3: Viability of *Hemileia vastatrix* spores after exposure to the CAs. Two concentrations of the solution were tested (1:100 and 1:200). Untreated spores (negative control), glutaraldehyde 2% / *p*-formaldehyde 4% (control for producing dead spores).

Untreated spores (control for viable spores) remained green after 72 hours, while spores treated with the CAs developed a concentration-dependent yellow coloration indicating an increase in the number of non-viable spores.

Accoriding to the results showed in the *in vitro* evaluations, the fungicidal activity of CAs was assessed in field. Weakly average (Tav), maximum (Tmax), minimum (Tmin) temperatures and relative humidity (RH) values recorded during the sampling period are presented in Figure 4.

Since the variability of the distribution (incidence) and the development (severity) of the disease was found very high in the same locality, the data obtained were normalized (Figures 5 and 6).

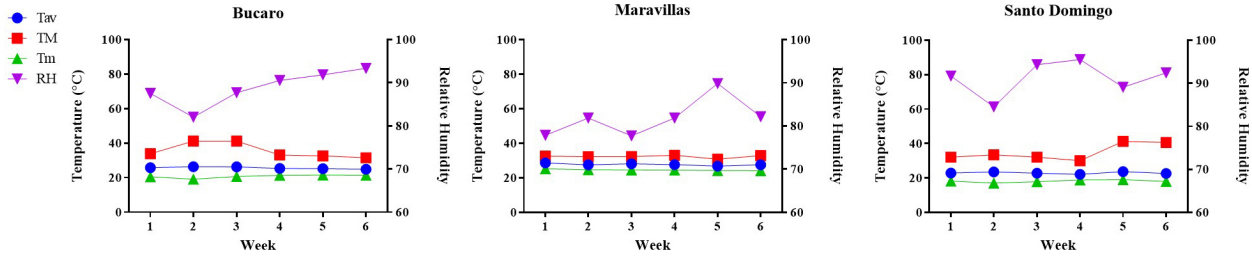


Figure 4: Weakly average (Tav), maximum (Tmax), minimum (Tmin) temperatures and relative humidity (RH) values recorded during the sampling period.

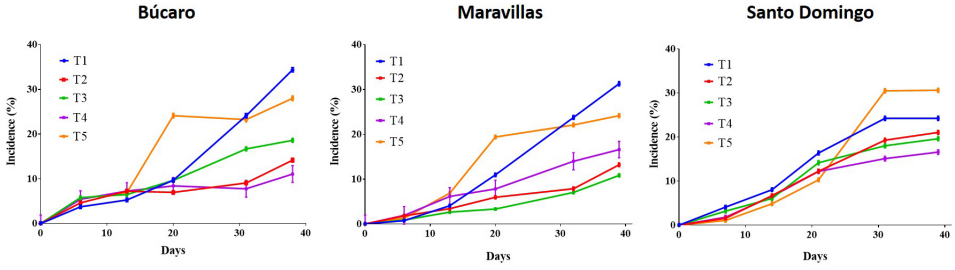


Figure 5: Normalized incidence of coffee rust expressed as percentage. Four treatments were tested (T1= control, T2= CAs 389 ppm, T3= CAs 584 ppm, T4= CAs 778 ppm, T5= copper oxychloride 5 000 ppm) in three locations (Maravillas, Santo Domingo and Búcaro).

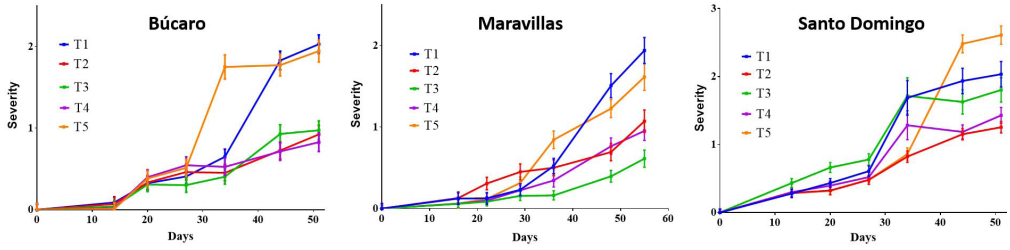


Figure 6: The severity of coffee rust infection expressed as the scale of damage of the leaves. T1= Control, T2= CAs 389 ppm, T3= CAs 584 ppm, T4= CAs 778 ppm, T5= Copper oxychloride 5000 ppm.

Significantly lower disease increase were observed for all the CAs treatments (T2-T4) in all three sites when compared to the control treatment as shown in the ANOVA anlysis (Supplementary information). However, during the application of copper based-fungicide an increase of the incidence was observed in the three locations. In particular, at Búcaro and Maravillas the disease for the copper treatments were significantly higher compared to those observed in untreated plants.

Copper phytotoxicity is suggested to be favored by higher relative humidity values as the ones observed during the sampling period at Búcaro and Maravillas (Ferreira et al., 2014). Slow drying conditions on the surface of the leaves can potentially lead to an increase in available copper ions involved in the production of harmful reactive oxygen species (Yruela, 2005). While going through this oxidative challenge, plants might be less able to cope with *H. vastatrix* infection. This phenomenon might explain the poor performance observed for the copper treatment at both sites.

As expected, given these differences, initial coffee rust incidences were also different. In Maravillas incidence averages were significantly lower when compared to the other two sites, ranging between 3 and 9%. In Santo Domingo, the observed incidence averages were between 10 and 16% of the total leaves sampled, while the incidence in Búcaro ranged from 16 to 22%. The lower starting incidence averages observed in Maravillas could have contributed to the more positive response to the treatments compared to the other two sites.

Shade conditions are relevant since the development of the disease is favored under longer periods of humidity and lower temperatures associated with dense coverage (López-Bravo et al., 2012). The rainfall was also monitored in Tapachula for the month of May and June which was found to be 396.0 and 514.6 mm respectively.

It is note worthy that in the Maravillas site, the evaluated CAs displayed greater inhibition of disease development than the copper and control treatments.

In Santo Domingo, lower minimal temperature and high humidity during the sampling period, probably favored the development of the rust. Although higher development rates were observed for every treatment in this site, the CAs in all tested concentrations performed better than the control and the copper treatments.

In general, the CAs treatments were more efficient than the copper treatment in slowing down the development of coffee rust under the tested conditions. This observation is particularly relevant in locations where preventive measures to avoid large scale infestations are not common practice, and fungicide treatments are applied when incidence values reach more than 5%. This is the case for many plantations in México and Central America, where application of fungicides following a yearly calendar is not a common practice due in part to the steep terrain and isolation of the coffee plantations (Avelino et al., 2015).

Intensity and severity measures were used to evaluate different aspects of *H. vastatrix* infection. While intensity, measured as the number of affected leaves from a sample, is a reflection of the different conditions affecting germination and leaf penetration, the evaluation of severity, as the proportion of the surface of infected leaves affected by the fungus, is a measure of the factors affecting the development and colonization (López-Bravo et al., 2012).

Although the two measurements are well correlated in the case of Coffee rust (Silva-Acuña; Zambolim, 1999), intensity of the infection at later phases of epidemics could play an important role on the increase of the development of *H. vastatrix*, which is expressed as severity (Waller, 1982).

We observed this phenomenon in our ANOVA analysis showed that the increase in severity on day 20 in Maravillas was significant. In Búcaro and Santo Domingo sites the severity increase was observed earlier, around day 15 (Figure 6).

The germination and colonization of *H. vastatrix* depend heavily on external factors particularly humidity, temperature, and light. (Kushalappa, 1979; Waller, 1982) The high observed humidity, especially at later sampling dates, favors germination, while optimal temperatures for both processes (21 °C-25 °C) are similar to the ones observed in Búcaro and Santo Domingo (Kushalappa, 1989; Waller, 1982).

The rate at which leaves are colonized also depends on internal factors of the plant. Since in all experimental sites, severity (proportion of the surface of infected leaves affected by the fungus) was consistently lower when the plants were treated with the CAs (Figure 6), it is possible that the tested CA also have a direct effect on plant metabolism. One interesting possibility would be the priming of the plant's defense mechanisms, an effect that has been observed for other CA (Aranega-Bou et al., 2014).

Organic agriculture began developing in the first half of the 20th century as an alternative to conventional production systems that use synthetic chemical inputs. Organic coffee is the largest category of US organic imports in terms of dollar value. Peru, Indonesia, Mexico, Brazil, and Colombia are the top five countries from which organic coffee is imported (Bailey, 2015). However, still the production of *organic coffee* is less than its demand. The most common disease control measure for organic coffee farming used nowadays are either by measures biological agents or copper fungicides. Nevertheless, disease control using live pathogens has several limitations. The foremost limitations are the presence of optimum temperature, humidity and light for the growth of the pathogens, while on the other hand use of bioactive compounds isolated from microorganisms has very few limitations and is more efficient. In this study we showed that biologically originated CA is much more proficient to control coffee rust disease (caused by *Hemileia vastatrix*) than commonly used copper fungicides. Hence, the use of CAs could definitely be promising for organic coffee cultivation.

CONCLUSIONS

The Carboxylic Acids (CA) produced by *Pichia membranifaciens* under culture conditions exhibited antifungal activity. The viability of *H. vastatrix* spores was affected by the CAs in a concentration-dependent manner. Under field conditions, the application of the CAs slowed down the rate of coffee rust progress, even in conditions where a commercial copper formulation performed poorly. The CAs is a natural and safe fungicide with the potential to be applied in the field to control *H. vastatrix*.

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REFERENCES

ARANEGA-BOU, P. et al. Priming of plant resistance by natural compounds. Hexanoic acid as a model. **Frontiers in Plant Science**, 5(488):1-12, 2014.

AVELINO, J. et al. The coffee rust crises in Colombia and Central America (2008-2013): Impacts, plausible causes and proposed solutions. **Food Security**, 7(2):303-321, 2015.

BAILEY, S. Keurig Green Mountain Caters to Organic Coffee Demand. Keurig Green Mountain's organic coffees. 2015. In: **Keurig Green Mountain Goes Organic with New Coffee Line**. Available in: <http://marketrealist.com/2015/07/keurig-green-mountain-caters-organic-coffee-demand/>. Access in: October 16, 2017.

CAO, S. et al. Improved control of anthracnose rot in loquat fruit by a combination treatment of *Pichia membranifaciens* with $CaCl_2$. **International Journal of Food Microbiology**, 126(1-2):216-220, 2008.

CAO, S. et al. Combination of *Pichia membranifaciens* and ammonium molybdate for controlling blue mould caused by *Penicillium expansum* in peach fruit. **International Journal of Food Microbiology**, 141(3):173-176, 2010.

CARRINL, G.; RICO-GRAY, V. Mycoparasites on the coffee rust in Mexico. **Fungal Diversity**, 11: 49-60, 2002.

CHAN, Z.; TIAN, S. Interaction of antagonistic yeasts against postharvest pathogens of apple fruit and possible mode of action. **Postharvest Biology and Technology**, 36:215-223, 2005.

CHEN, C. Y.; SÉGUIN-SWARTZ, G. A rapid method for assessing the viability of fungal spores. **Canadian Journal Plant Pathology**, 232:230-232, 2002.

CONAGUA. **Informacion climatologica por estado**. 2016. Available in: <http://smn.cna.gob.mx/es/informacion-climatologica-ver-estado?estado=chis>. Access in: December, 5, 2016.

CRISTANCHO, M. A. et al. Annotation of a hybrid partial genome of the coffee rust (*Hemileia vastatrix*) contributes to the gene repertoire catalog of the Pucciniales. **Frontiers in Plant Science**, 5(594):1-11, 2014.

CSUTAK, O. et al. Antagonistic activity of three newly isolated yeast strains from the surface of fruits. **Food Technology and Biotechnology**, 51(1):70-77, 2013.

DE SOUZA, A. F. et al. Chemical approaches to manage coffee leaf rust in drip irrigated trees. **Australasian Plant Pathology**, 40(3):293-300, 2011.

EDWARDS, K. et al. A simple and rapid method for the preparation of plant genomic DNA for PCR analysis. **Nucleic Acids Research**, 19(6):1349, 1991.

EPPO. Design and analysis of efficacy evaluation trials. **EPPO Bulletin**, 42(3):367-381, 2012.

FAN, Q. et al. Postharvest biological control of *Rhizopus* rot of nectarine fruits by *Pichia membranifaciens*. **Plant Disease**, 84(11):1212-1216, 2000.

FAO. FAOSTAT. **Crops and livestock products**. 2016. Available in: <http://www.fao.org/faostat/en/#data/TP/visualize>. Access in: December, 5, 2016.

FERREIRA, L. C. et al. Copper oxychloride fungicide and its effect on growth and oxidative stress of potato plants. **Pesticide Biochemistry and Physiology**, 112:63-69, 2014.

GEORGOPOULOS, P. G. et al. Environmental copper: Its dynamics and human exposure issues. **Journal of Toxicology and Environmental Health Part B**, 4(4):341-394, 2001.

GILLER, K. E. et al. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: A review. **Soil Biology and Biochemistry**, 30(10-11):1389-1414, 1998.

GOUVEIA, M.; MANUELA, C. et al. Genetic diversity in *Hemileia vastatrix* based on RAPD markers. **Mycologia**, 97(2):396-404, 2005.

HADDAD, F. et al. Isolation and selection of *Hemileia vastatrix* antagonists. **European Journal of Plant Pathology**, 139(4): 763-772, 2014.

HASSAN, R. et al. Effect of some organic acids on some fungal growth and their toxins production. **International Journal of Advances in Biology**, 2(1):1-11, 2015.

HESTER, S. et al. The hepatocarcinogenic conazoles: Cyproconazole, epoxiconazole, and propiconazole induce a common set of toxicological and transcriptional responses. **Toxicological Sciences**, 127(1):54-65, 2012.

HSIAO, C. P.; SIEBERT, K. J. Modeling the inhibitory effects of organic acids on bacteria. **International Journal of Food Microbiology**, 47(3):189-201, 1999.

HU, W.; MURATA, K.; ZHANG, D. Applicability of LIVE/DEAD BacLight stain with glutaraldehyde fixation for the measurement of bacterial abundance and viability in rainwater. **Journal of Enviromental Science**, 51:202-213, 2017.

INSTITUTO NACIONAL DE ESTADÍSTICA Y GEOGRAFÍA - INEGI. **Anuario estadistico y geografico de Chiapas**, 2015. Available in: <http://www.diputados.gob.mx/sedia/biblio/usieg/usieg_anu_est16/Chiapas/Aspectos%20geográficos.xls>. Access in: October 16, 2017.

JACKSON, D. et al. Indirect biological control of the coffee leaf rust, *Hemileia vastatrix*, by the entomogenous fungus *Lecanicillium lecanii* in a complex coffee agroecosystem. **Biological Control**, 61(1):89-97, 2012.

KUSHALAPPA, A. C. Advances in coffee rust research. **Phytopathology**, 27(57):503-531, 1989.

LIU, S. et al. Biological control of phytopathogenic fungi by fatty acids. **Mycopathologia**, 166(2): 93-102, 29 2008.

LÓPEZ-BRAVO, D. F. et al. Shade is conducive to coffee rust as compared to full sun exposure under standardized fruit load conditions. **Crop Protection**, 38:21-29, 2012.

LUTZ, M. C. et al. Efficacy and putative mode of action of native and commercial antagonistic yeasts against postharvest pathogens of pear. **International Journal of Food Microbiology**, 164(2-3):166-172, 2013.

MCCOOK, S.; VANDERMEER, J. The big rust and the red queen: Long-term perspectives on coffee rust research. **Phytopathology**, 105(9):1164-1173, 2015.

NORMA OFICIAL MEXICANA NOM-032-FITO-1995, Por la que se establecen los requisitos y especificaciones fitosanitarios para la realización de estudios de efectividad biológica de plaguicidas agrícolas y su dictamen técnico. **Diario Oficial de la Federación**. 1995.

NUNES, C. C. et al. Genetic diversity of populations of *Hemileia vastatrix* from organic and conventional coffee plantations in Brazil. **Australasian Plant Pathology**. 38(5):445-452, 2009.

PEEL, M. C. et al. Updated world map of the Köppen-Geiger climate classification. **Hydrology and Earth System Sciences**, 11(5):1633-1644, 2007.

POHL, C. H. et al. Antifungal free fatty acids: A review. In: Méndez-Vilas, A. (ed). **Science against microbial pathogens: Communicating current research and technological advances**. Spain: Formatex. v. 3. 2011. p.61-71.

RANKINEI, B. C. *Pichia membranaefaciens*, a yeast causing film formation and off-flavor in table wine. **The American Journal of Enology and Viticulture**, 17:82-86, 1964.

ROMPRÉ, A. et al. Detection and enumeration of coliforms in drinking water: Current methods and emerging approaches. **Journal of Microbiological Methods**, 49(1):31-54, 2002.

SCHOCH, C. L. et al. Nuclear ribosomal internal transcribed spacer (ITS) region as a universal DNA barcode marker for Fungi. **Proceedings of the National Academy of Sciences of the United States of America**, 109(16):1-6, 2012.

SENASICA, **Roya del Cafeto Hemileia Vastatrix Berkeley & Broome**. Ficha Técnica No. 2016. 40. Dirección General de Sanidad Vegetal. Available in: <http://www.cesaveson.com/files/docs/campanas/vigilancia/fichas2016/ROYAcafeto.pdf> Access in: October 16, 2017.

SILVA-ACUÑA, R.; ZAMBOLIM, L. Incidence-severity relationships in the pathosystem *Coffea arabica-Hemileia vastatrix*. **Plant Disease**, 83(2):186-188, 1999.

STEEL, R. G. D.; TORRI, J. H. **Principles and procedures of statistics**: A Biometrics Approach, 2. ed. McGraw-Hill, New York, 1997. pp 86-167.

TOJU, H. et al. High-coverage ITS primers for the DNA-based identification of ascomycetes and basidiomycetes in environmental samples. **PLoS ONE**, 7(7):1-11, 2012.

WALLER, J. M. Coffee rust-epidemiology and control. **Crop Protection**, 1(4):385-404, 1982.

YRUELA, I. Copper in plants. **Brazilian Journal of Plant Physiology**, 17(1):145-156, 2005.

ZAMBOLIM, L. Current status and management of coffee leaf rust in Brazil. **Tropical Plant Pathology**, 41(1):1-8, 2016.