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RESEARCH PAPER

Uredospore germination of *Hemileia vastatrix* and its inhibition by the effect of plant extracts *in vitro*

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Abstract

M.A. Morales-Antonio, G.M. Santiago-Martínez, A. Vásquez-López, G. Rodríguez-Ortiz, D. Soto-Castro, S. Lozano-Trejo, and E. Castañeda-Hidalgo. 2021. Uredospore germination of *Hemileia vastatrix* and its inhibition by the effect of plant extracts *in vitro*. Int. J. Agric. Nat. Resour. 108-114. Coffee leaf rust, caused by *Hemileia vastatrix*, is the most important disease of coffee (*Coffea arabica* L.) in Mexico. It causes production losses of up to 40% and leads to the use of considerable volumes of synthetic fungicides. The main goals of this research were to identify the temperature, pH, incubation time, and luminosity required for *in vitro* germination of *H. vastatrix* uredospores and to evaluate the effect of plant extracts on their germination. Uredospores from coffee plants (var. Caturra) grown in the state of Oaxaca, Mexico, were collected and subjected to treatments consisting of combined levels of T°, pH, and incubation time. The treatments were evaluated in darkness and under low-intensity white light (15 W). Uredospore germination occurred in the absence and presence of light. The highest percentage of uredospore germination was 44.95%, which occurred at pH 5.7, between 18 and 24 °C, and with an incubation time of 24 h. The effect of 30 plant extracts was evaluated in terms of inhibition of uredospore germination. The acetone and ethanol extracts of *Tribulus terrestris*, *Datura ferox, Mansoa alliacea*, *Ricinus communis,* and *Acacia farnesiana* inhibited 100% of uredospore germination. Thus, plant extracts may contribute to the integrated disease management of coffee leaf rust.

Key words: *Coffea arabica*, coffee leaf rust, organic fungicides, secondary metabolites.

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Introduction

Mexico is among the top 10 coffee bean (*Coffea arabica* L.) producing countries. In 2018, production reached 860,000 t of coffee beans out of an area of 630,000 ha, and the state of Oaxaca contributed 20% of the national production (SADER, 2018). Coffee production is affected by leaf rust caused by *Hemileia vastatrix*, a fungus that reduces production up to 40%. In Mexico, varieties susceptible to this disease, such as Typica, Bourbon, Mundo Novo, Caturra, Garnica, Catuaí, and Pluma Hidalgo, are grown (Hernández-Martínez & Velázquez-Premio, 2016). Uredospores of *H. vastatrix* are the primary inoculum of the disease cycle. Under *in vitro* conditions, uredospores germinate at minimum, optimal and maximum temperatures of 15.5, 22.0, and 28.5 °C, respectively, in darkness and under low-intensity light (up to 25 luxes) and high humidity (>95%). Uredospore germination of *H. vastatrix* starts after 2.6 h and stops before 8 h under *in vitro* conditions (Nutman *et al*., 1963; Diniz *et al*., 2012; Avelino *et al*., 2015).

The incidence of coffee leaf rust has been mitigated using resistant varieties, biological control, and cultural labor. The most common method to reduce leaf rust is spraying synthetic fungicides, which increases production costs and ecological risks (Avelino & Rivas, 2013; Hernández-Martínez & Velázquez-Premio, 2016). Leaf rust affects the economy and threatens the food security of Mexican families whose income depends on coffee. In Mexico, in the last four years, the area cultivated with coffee decreased by approximately 110,000 ha, with leaf rust being an important factor in this loss (SADER, 2018). In this context, it is important to look for alternatives that complement integrated crop management. One of them is the use of plant extracts (Rodríguez *et al*., 2000), which consist of a mixture of biomolecules with antifungal properties derived from the secondary metabolism of plants (Reddy *et al*., 2020). The aims of this study were to identify the optimal temperature, pH, incubation time and luminosity for the germination of *H. vastatrix* uredospores and to evaluate the effect of plant extracts on *H. vastatrix* uredospore germination under *in vitro* conditions.

Materials and methods

Factors that affect uredospore germination

Hemileia vastatrix uredospores were collected from 35-year-old coffee plants (*Coffea arabica* L.; var. Caturra) in a plot belonging to the Instituto Tecnológico del Valle de Oaxaca (17°01'N, 96°46'W, 1,567 m altitude) in 2018. A completely randomized design was used with an 8 x 3 x 4 factorial array (T°, pH, time): incubation temperatures (18, 20, 22, 24, 26, 28, 30, and 32 °C), levels of pH (3.7, 5.7, and 7.7) in the substrate (potato-dextrose-agar, BD Bioxon™, Mexico) and incubation times (3, 6, 12, and 24 h), all independently evaluated in two light environments (darkness and 15 W white light). Microphotographs were taken of the uredospores of each treatment with a digital camera (AxioCam ICc1) mounted on a compound microscope (Carl Zeiss, Primo Star). In each image, 3000 uredospores were selected, and the number of germinated spores was recorded. The percentage of germinated uredospores was estimated with the formula $Ug = [(G_i)\ (100)] / T_i$, where Ug = percentage of germinated uredospores and G_i = number of germinated uredospores in the experimental treatment at time *i*. T_i = total uredospores examined in the experimental treatment at time *i*. The data were transformed to $e^{\sqrt{x+1}}$ to fulfil normality (Shapiro–Wilks) and homogeneity of variance (Bartlett). An analysis of variance and separation of means (Duncan, 0.05) were performed with the Statistical Analysis System program 9.0 (SAS Institute, Inc., Cary, NC).

Effect of plant extracts on uredospore germination

The effects of extracts from 10 plant species were evaluated (Table 1). The plants were collected in the state of Oaxaca, Mexico, in 2018. The plants

were washed with water and dried in the shade (22 °C ± 2 °C) for 30 days. Ethanol and acetone extracts were obtained by mixing 25 g of dry material with 350 mL of distilled water, absolute ethanol or acetone. The mixture was placed in an ultrasound sonicator bath (Branson Model 3800) for 3 h. The solvent was removed with a rotavapor (Buchi Model B-100) at 40 °C. Ninety treatments resulting from the interaction of 10 types of plant extracts, three solvents (acetone, ethanol, and water) and three concentrations (0.5, 1.0, and 2.0%) were established; each was replicated five times. PDA was prepared (pH 5.7) and independently mixed with each of the dosages of the plant extracts. The PDA was poured into Petri dishes, and after 24 h, between 3000 and 3500 uredospores were placed on the media. The dishes were incubated at 23 °C in constant darkness for 6 h. An absolute control (PDA culture medium without extract) was included. Six hours after seeding, microphotographs were taken of the uredospores of each treatment with a digital camera (AxioCam ICc1) mounted on a compound microscope (Carl Zeiss, Primo Star). In each image, 3000 uredospores were selected, and the number of germinated spores was recorded. The percentage of inhibition of uredospore germination was estimated with the formula:

$$I = \frac{C-D}{T}\ (100)$$

where I = percentage of non-germinated uredospores; C = number of germinated uredospores in the control treatment at time *i*; and T = number of germinated uredospores in the experimental treatment at time *i*. A completely randomized experimental design was used with a 10×3×3 (plant extracts, solvents, and concentrations) factorial array. An analysis of variance was performed with variables transformed to $e^{(x/100)}$ and separation of means (Duncan, 0.05) with the Statistical Analysis System program 9.0 (SAS Institute, Inc., Cary, NC).

Results and discussion

Factors involved in uredospore germination

The factors (T°, pH, and incubation time), evaluated in darkness and in the presence of light, had a significant ($p<0.01$) influence on the germination of *H. vastratrix* uredospores (Table 2). In darkness, the highest percentage of uredospore germination was between 41.70 and 44.95%, which was favored at pH 5.7, under 24 h of incubation time and at T° between 18 and 24 °C. In the presence of light, the highest percentage of uredospore germination was between 42.92 and 43.41% and occurred at pH 5.7, with a 24 h incubation time and at T° between 18 and 20 °C (Table 3). An acidic (3.3) or alkaline (7.7) pH, as well as

Table 1. Plants collected in Oaxaca, Mexico, in 2018 used to extract organic compounds with ethanol, acetone and water.

Scientific name	Common name	Family	Organ
Acacia farnesiana	Huisache	Fabaceae	Leaves and flower
Allium sativum	Garlic	Amaryllidaceae	Fruit
Anredera vesicaria	Sacasile	Basellaceae	Root
Cestrum sp.	Night blooming jasmine	Solanaceae	Leaves
Datura ferox	Fierce thorn apple	Solanaceae	Leaves and seed
Mansoa alliacea	Wild garlic	Bignoniaceae	Leaves
Ricinus communis	Castor oil plant	Euphorbiaceae	Seeds
Thymus vulgaris	Thyme	Lamiaceae	Leaves
Tribulus terrestris	Caltrop	Zygophyllaceae	Leaves and flowers
Zingiber officinale	Ginger	Zingiberaceae	Root

temperatures over 24 °C, did not contribute to uredospore germination (Table 3). These results agree with those by Nutman *et al*. (1963) and Diniz *et al*. (2012), who reported that, under *in vitro* conditions, uredospore germination occurs in darkness or in the presence of low-intensity light (up to 25 luxes) at minimum, optimal and maximum T° values of 15.5, 22.0, and 28.5 °C, respectively. These authors stated that in conditions of darkness, germination starts after 2.6 h and stops before 8 h after inoculation. However, in our study, we found that uredospore germination occurs at 2 h until 24 h after inoculation. Not all fungal uredospores germinate; germination depends on the age. Mature uredospores have a higher rate of germination, but this rate decreases exponentially as they age (Nutman *et al*., 1963; Arroyo *et al*., 2019).

Table 2. Significance of squared means for germination and inhibition of *Hemileia vastatrix* uredospores under two conditions of light (low-intensity light and continuous darkness).

SV	DF	Light condition		SV	DF	Germination inhibition
		Light¶	Dark¶			
T°	7	241768.8**	769386.9**	Solvent (D)	2	0.389**
pH	2	590960.1**	5452402.4**	Plant (V)	9	0.733**
Time (t)	3	216788.3**	995499.6**	Concentration (C)	2	2.088**
T°×pH	14	232393.7**	758082.0**	D×V	18	0.292**
T°×t	21	93305.7**	136626.0**	V×C	18	0.356**
pH×t	6	205869.4**	948356.5**	D×C	4	0.012ns
T°×pH×t	42	90294.1**	134582.5**	D×V×C	36	0.107**
Error	648	4048.9	37581.6**	Error	810	0.006
Total	743			Total	899	

SV = source of variation, DF = degree of freedom, T° = temperature (18, 20, 22, 24, 26, 28, 30, and 32 °C), pH (3.7, 5.7, and 7.7), and t = incubation time (3, 6, 12, and 24 h). Variables transformed to ¶ $e^{\sqrt{x+1}}$ and ¶¶$e^{(x/100)}$. **Highly significant (P<0.01) and ns: Not significant (P>0.05).

Table 3. Germination (%) of *Hemileia vastatrix* uredospores as a function of temperature, pH, and incubation time under artificial light and darkness.

Temperature (°C)	Dark				Light			
20	11.64	±	1.69	a	8.69	±	1.30	a
22	11.38	±	1.84	a	1.80	±	0.34	b
24	10.96	±	1.69	a	0.44	±	0.03	e
18	9.36	±	1.52	b	8.93	±	1.56	a
26	2.89	±	0.39	c	1.32	±	0.22	bc
30	0.75	±	0.09	d	0.69	±	0.10	de
28	0.56	±	0.09	d	0.39	±	0.13	e
32	0.35	±	0.06	d	1.14	±	0.18	cd
pH	Dark				Light			
5.7	16.36	±	1.16	a	7.49	±	0.81	a
7.7	1.79	±	0.16	b	1.19	±	0.13	b
3.7	0.36	±	0.03	c	0.27	±	0.02	c
Time	Dark				Light			
24	9.58	±	1.18	a	5.76	±	0.88	a
12	8.22	±	1.10	b	3.94	±	0.67	b
6	5.61	±	0.84	c	1.60	±	0.26	c
3	1.26	±	0.23	d	0.63	±	0.14	d

Means are ± standard error. Different letters in each factor indicate statistically significant differences (Duncan, α = 0.05).

Effect of plant extracts on uredospore germination

The plant extracts evaluated in this study had a significant effect (P<0.01) on uredospore germination (Table 2). Ethanolic and acetone extracts of *T. terrestris*, *A. vesicaria*, *A. farnesiana*, *Cestrum* sp., *R. communis*, *D. ferox, M. alliacea* and *Z. officinale*, at 0.5%, inhibited more than 95% germination. The same results were obtained with aqueous extracts of *T. terrestris*, *A. sativum*, *A. vesicaria*, *A. farnesiana*, *Cestrum* sp. and *R. communis*, at 0.5%; *Z. officinale,* at 1.0%; and *M. alliacea* and *T. vulgaris,* at 2.0% (Figure 1). The biomolecules that plants produce during secondary metabolism are terpenes, phenols, quinones, polyacetylenes, polyenes, alkamides, carbohydrates, organic acids, alkaloids, nonprotein amino acids, glucosinolates, lectins or peptides, which alter the composition of the cell wall and membrane and modify the basic functions of the cells (Silva & Fernandes, 2010; Reddy *et al*., 2020). The essential oils and extracts of the plants used in this study have shown antifungal activity against phytopathogenic fungi in previous studies (Silva & Fernandes, 2010), but there is no evidence that they inhibit the growth and development of *H. vastatrix, e*xcept for *T. vulgaris*, which has previously been reported as an inhibitor of uredospore germination of this fungus (Borges *et al*., 2012).

It is concluded that uredospores of *H. vastratrix* can germinate in darkness and in the presence of low-intensity light (15 W) at an optimal temperature of 18 and 24 °C and pH 5.7. Germination started before 3 h and stopped 24 h after incubation. The plant extracts of *A. farnesiana*, *A. sativum*, *A. vesicaria*, *Cestrum* sp., *D. ferox*, *M. alliacea*, *R. communis*, *T. vulgaris*, *T. terrestris* and *Z. officinale* reduced the germination of *H. vastratrix* uredospores by 95% under *in vitro* conditions. This study provides basic information on the capacity of plant extracts evaluated to inhibit germination of *H. vastratrix* uredospores. This study may contribute to the selection of candidate compounds for the development of new selective natural fungicides for the management of plant diseases in agricultural crops.

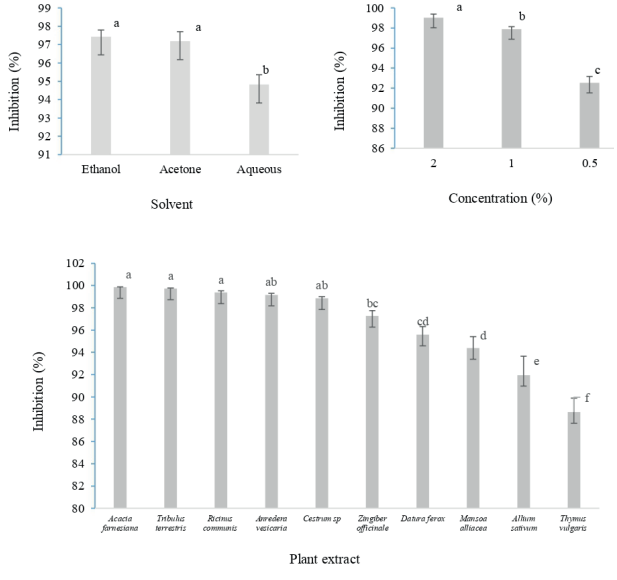

Figure 1. Inhibition (%) of *H. vastatrix* uredospore germination by the effect of organic compounds extracted with acetone, ethanol, and water from ten plant species collected in the state of Oaxaca, Mexico, in 2018. Vertical lines at the top of the bars represent standard error. Different letters in each factor indicate statistically significant differences (Tukey, $\alpha = 0.05$)

Resumen

M.A. Morales-Antonio, G.M. Santiago-Martínez, A. Vásquez-López, G. Rodríguez-Ortiz, D. Soto-Castro, S. Lozano-Trejo, y E. Castañeda-Hidalgo. 2021. Germinación de uredosporas de *Hemileia vastatrix* y su inhibición por efecto de extractos vegetales *in vitro*. Int. J. Agric. Nat. Resour. 108-114. La roya, causada por *Hemileia vastatrix*, es la enfermedad más importante del cultivo de café (*Coffea arabica* L.) en México; ésta ocasiona pérdidas de producción de hasta 40 % y uso de volúmenes considerables de fungicidas sintéticos. Los objetivos de esta investigación fueron identificar la temperatura, pH, tiempo de incubación y luminosidad que inciden en la germinación de uredosporas *in vitro*; y evaluar el efecto de extractos vegetales sobre su germinación. Se recolectaron uredosporas de plantas de café var. Caturra, cultivadas en el estado de Oaxaca, México, y se sometieron a tratamientos combinando niveles de T°, pH y tiempo de incubación. Los tratamientos se evaluaron en oscuridad y en luz blanca de baja intensidad (15 W). Las uredosporas germinaron en ausencia y en presencia de luz. La germinación máxima fue de 44.95 % y ocurrió en pH de 5.7, temperaturas entre 18 y 24 °C y tiempo de incubación de 24 h. Se evaluaron 30 extractos vegetales sobre la inhibición de germinación de uredosporas. Los extractos de *Tribulus terrestris*, *Datura ferox, Mansoa alliacea*, *Ricinus communis* y *Acacia farnesiana*; extraídos con acetona y etanol; inhibieron en 100 % la germinación de uredosporas. Los extractos vegetales pueden contribuir al manejo integrado de la roya en café.

Palabras clave: *Coffea arabica*, fungicidas orgánicos, metabolitos secundarios, roya del café.

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