Antifungal Potential of Crude Plant Extracts on Conidial Germination of Two Isolates of *Colletotrichum gloeosporioides* (Penz.) Penz. and Sacc.

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Abstract. Crude plant extracts of leaves and stems of 40 different plant species from the state of Morelos, Mexico, were used to compare conidia germination of two isolates of *Colletotrichum gloeosporioides* obtained from papaya fruit with anthracnose symptoms, collected from the states of Guerrero and Veracruz, Mexico. In general, better antifungal effect was observed with crude plant extract of night-blooming jessamine (*Cestrum nocturnum*) and cherimoya (*Annona cherimola*) for both isolates, after 14 or 18 h incubation, determined by optical microscopy method (OMM) and espectrophotometry method (EM). Conidia of both fungal isolates incubated in crude extracts of *Origanum majorana*, *Carica papaya*, *Citrus aurantium*, *Citrus aurantifolia*, *Bougainvillea spectabilis*, *Justicia spicigera*, *Petroselinum sativum*, *Parthenium hysterophorus*, *Schinus molle*, and *Ricinus communis*, showed less than 61% germination by OMM and less than 56% by EM. The remaining 28 crude extracts did not show antifungal effect. Conidial germination varied according to extract, isolate, time of incubation, and methodology evaluated.

Additional keywords: Natural compounds, spectrophotometry, anthracnose.

Resumen. Extractos crudos de hojas y tallos de 40 especies diferentes de plantas del estado de Morelos, México, se utilizaron para comparar la germinación conidial de dos aislamientos de *Colletotrichum gloeosporioides*, los cuales se obtuvieron de frutos de papaya con síntomas de antracnosis, provenientes del estado de Guerrero y Veracruz, México. En general, el mejor efecto antifúngico se observó con el extracto crudo de huele de noche (*Cestrum nocturnum*) y chirimoya (*Annona cherimola*) para ambos aislamientos después de 14 ó 18 h de incubación y determinado por el método de microscopía óptica (MMO) y el método de espectrofotometría (ME). Los conidios de ambos aislamientos incubados en los extractos crudos de *Origanum majorana*, *Carica papaya*, *Citrus aurantium*, *Citrus aurantifolia*, *Bougainvillea spectabilis*, *Justicia spicigera*, *Petroselinum sativum*, *Parthenium hysterophorus*, *Schinus molle* y *Ricinus communis* mostraron porcentajes de germinación menores de 61% por MMO y menores de 56% por ME. Los 28 extractos crudos restantes no mostraron algún efecto antifúngico. La germinación de conidios varió de acuerdo al extracto vegetal, aislamiento, tiempo de incubación y método de evaluación.

Palabras clave adicionales: Compuestos naturales, espectrofotometría, antracnosis.

Colletotrichum gloeosporioides (Penz.) Penz. y Sacc. is the casual agent of anthracnose, an important disease of papaya fruit (*Carica papaya* L.) (Alvarez and Nishijima, 1987; Snowdon, 1990). Bolkan *et al*. (1976) reported that rots originated by *C. gloeosporioides* isolated from immature and mature fruit accounted for 40 and 89% of the infection, respectively. Chemical control which is used to reduce incidence of postharvest diseases in papaya is causing the development of fungal resistance to chemical products (Brent and Hollomon, 1998). Application of higher concentrations of chemicals in an attempt to overcome anthracnose disease, increases the risk of high levels of toxic residues, which is particularly serious since papaya fruit is consumed in a relatively short time after harvest. The exploitation of natural products such as plant extracts is believed to be safer to consumers and the environment. In the state of Morelos, Mexico, the antimicrobial properties of plant extracts from various species have been proven to control fungal development either *in vitro* or *in vivo*. In *vitro* studies carried out by Bautista-Baños *et al*. (2000a) reported that among 19 different botanical species tested, the aqueous extract of leaves of custard apple (*Annona reticulata* L.) and papaya among various others, inhibited spore formation and germination of *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. Conidial inhibition was also observed when *C. gloeosporioides* was grown on these two extracts (Bautista-Baños *et al*., 2002).

Current methods used to evaluate the efficiency of plant extracts for control of fungi include the use of high quantity of materials such as nutrient media and Petri plates, which in addition, are time consuming and expensive. Wilson *et al*. (1997) tested the microtiter method to evaluate the antifungal activity of crude plant extracts against *Botrytis cinerea* Pers.:Fr., highlighting the efficacy of this method. Then, spectrophotometry´s method was compared at the same time with the microscopy´s method to evaluate spore germination (counting under light microscopy). The objective of this research was to evaluate the effect 40 crude plant extracts on conidial germination of *C. gloeosporioides* by optical microscopy and espectrophotometry.

Isolates. *Colletotrichum gloeosporioides* was isolated from diseased papaya fruit showing anthracnose symptoms, harvested in the states of Veracruz and Guerrero, Mexico. Pure monosporic cultures were maintained on potato-dextrose-agar (PDA). To maintain pathogenicity of the fungus, periodic inoculations and reisolations from infected papaya were carried out. The parameters with significantly differences between Veracruz and Guerrero isolates were: mycelial growth, growth rate, and germination in different growing media. Experiments were carried out using 10-15 days old cultures.

Plant material. Plant selection was according to the fungicidal or bactericidal background previously reported by Flores-Moctezuma *et al*. (2002). Forty different plant species corresponding to various plant families including medicinal, forest, bush, fruit, and vegetable plants were field collected or bought at local markets from different regions of the state of Morelos (Table 1). Plant species were collected throughout the year.

Plant material preparation. 100 g of each of the collected plant material were disinfested in 2% sodium hypochlorite for 15 min, rinsed twice in distilled water for 15 min and air-dried at 26ºC for 3 to 4 h depending on the plant specie. Materials were then placed in polyethylene bags and frozen at -20ºC for a minimum of 12 h. Plant materials were thawed at ambient temperature for 1 h, wrapped in a fine cotton fabric and pressed to obtain the extracts (freezing and thawing fracture plant cells allowing the collection of fluids free of tissue); then, they were centrifuged (RC-3B Refrigerated Centrifuge, Sorvall Instruments) at 4500 x g for 30-60 min at 19ºC. Supernatants were filter sterilized (0.22 µm Millipore, MA, USA) and used as test extracts. Extracts were kept in sterile amber bottles at 4ºC for a maximum of 60 h.

Preparing the multi-well plates according to the spectrophotometer and the optical microscopy method. 30 µl of a 10% solution of the crude sterile extract and an inactive conidial suspension of 2 x 10^5 (270 µl) in sterile water, were placed into each well of a row of a 96 multi-well microtritation plate. The inactive conidial suspension was obtained by thermic shock (5 min at 100°C and 15 min at 4°C). Plant extracts with active conidial suspension were added to a second multi-well plate. Controls consisted of a row containing sterile water with a conidial suspension from Veracruz or Guerrero which presented high percent spore germination in water. Five extracts with six replicates were able to be analysed for each isolate. After 14 and 18 h incubation of the plates at 28°C (before 14 h incubation there was not significant differences in spore germination), the absorbancy of fungal growth in the wells was measured with a Labsystems Multiscan EX (Model 355) microplate reader at wavelength 492 nm which was used by Wilson *et al*. (1997). This method scans a first plate of plant extracted material with the inactive conidial suspension. The second plate reading contains the plant extract with the active conidial one. The percent spore germination by the espectrophotometry method (EM) was determined with the following equations: % GIAC = (AIC) (100% GIIC)/AAC and % GAC = 100-% GIAC, where: % GIAC = percent germination inhibition of active conidia, % GIIC = percent germination inhibition of inactive conidia, % GAC = percent germination of active conidia, AIC = absorbancy of inactive conidia, AAC = absorbancy of active conidia. A third multi-well plate (plant extracts with an active conidia suspension) was prepared and 50 µl aliquot of this suspension was placed on a 20-mm diameter agar disk and after 14 and 18 h at 28ºC stained with lactophenol acid fuchsin. The percent conidial germination was determined at 40X (optical microscopy method = OMM). Plant extracts were tested separately in lots of five and a control. The entire study consisted of eight experiments.

Statistical analysis. Percentage conidial germination for EM and OMM were analysed using the statistical program Sigma Stat 2.0 using test of Kruskal-Wallis to obtain mean separation ($p = 0.05$).

In the isolate from Veracruz, germination measured by EM was significantly lower ($p = 0.05$), (13-45%) than control (1-8%) after 14 and 18 h respectively (Table 2, Lot 1). In general, the isolate of *C. gloeosporioides* from Guerrero and treated with extracts of *Bougainvillea spectabilis*, *Annona muricata*. *Citrus aurantifolia* and *C. aurantium* had percentage conidial germination equal to that of the control in a range of 11 to 18%. In lot 2, in both isolates treated with plant extract of *C. nocturnum* was seen the best fungicidal effect (1-5% germination) ($p = 0.05$) after both incubation periods determined by OMM and EM. None of the isolates of *C. gloeosporioides* was controlled when treated with plant extracts of *Persea americana*, *Ficus nitida*, *Psidium guajava* and *Mangifera indica* at both incubation times. Percent germination at 14 and 18 h of incubation in both isolates treated with *Pithecellobium dulce*, *Mentha* x *piperita*, *Piper auritum* and *Passiflora edulis* were significantly different ($p = 0.05$) than germination of the untreated isolates by EM (Lot 3). However, *P. edulis* showed the best fungicidal effect against *C. gloeosporioides* in the isolate from Veracruz after both incubation periods by EM. In Lot 4, conidial germination of *C. gloeosporioides* from Veracruz was significantly lower (56-64%) ($p = 0.05$) than control treatment (81%) after 14 h with extracts of *Ricinus communis*, *Parthenium*

Table 1. Family, cientific and common name, and type of organ of the selected plant species.

Plant family	Scientific name	Common name	Plant organ
Acantaceae	*Justicia spicigera* Schult.	Mexican honeysuckle	L
Anarcadiaceae	*Mangifera indica* L.	Mango	L
	Schinus molle L.	Pepper tree	L
Anonaceae	*Annona cherimola* Mill.	Cherimoya	L
	Annona muricata L.	Soursop	L
	Annona reticulata L.	Custard apple	L
Apiaceae	*Petroselinum savitum* (Mill.) Nyman ex A.W. Hill	Parsley	L&S
Brassicaceae	*Brassica oleracea* L.	Cabbage	L
Caricaceae	*Carica papaya* L.	Papaya	L
Compositae	*Barkleyanthus salicifolius* (Kunth) H.E. Robins and Brett.	Willow ragwot	L&S
	Parthenium hysterophorus L.	Ragweed	L
Chenopodiaceae	*Chenopodium ambrosioides* L.	Epazote	L
Ebenaceae	*Diospyros ebenaster* Retz	Black sapote	L
Ericaceae	*Arctostaphylos polifolia* Humboldt, Bompland and Kunth.	Mexican manzanita	L
Euphorbiaceae	*Ricinus communis* L.	Castorbean	L
Fabaceae	*Erythrina americana* Mill.	Naked coral tree	L
	Inga jinicuil Schltdl. and Cham. ex G.	Ice-cream beans	L
	Pithecellobium dulce (Roxb.) Benth	Huamuchil	L
Labitae	*Mentha* x *piperita* L.	peppermint	L&S
	Ocimum basilicum L.	Basil	P
	Origanum majorana L.	Sweet marjoram	P
	Rosmarinus officinalis L.	Rosemary	P
	Thymus vulgaris L.	Thyme	P
Lauraceae	*Persea americana* Mill.	Avocado	L
Liliaceae	*Allium schoenoprasum* L.	Chives	L
Meliaceae	*Azadirachta indica* Adv. Juss.	Neem	L
Mirtaceae	*Eucaliptus* spp.	Eucalyptus	L
	Psidium guajava L.	Guava	L
Moraceae	*Ficus nitida* Thumb.	Indian laurel	L
Nigtaginaceae	*Bougainvillea spectabilis* Willd., B. glabra Choisy	Bouganbilia	L
Passifloraceae	*Passiflora edulis* Sims.	Passion fruit	L
Piperaceae	*Piper aduncum* L.	Spiked pepper	L
	Piper auritum Kunth	Mexican pepperleaf	L
Poaceae	*Cymbopogon citratus* (DC ex Nees) Stapf	Lemon grass	L
Punicaceae	*Punica granatum* L.	Pomegranate	L
Rutaceae	*Citrus aurantifolia* (Christm.) Swingle	Mexican lime	L
	Citrus aurantium L.	Bitter orange	L
	Ruta chalepensis L.	Fringed rue	P
Solanaceae	*Cestrum nocturnum* L.	Night-blooming jessamine	L
	Nicotiana glauca Graham	Wild tobacco	L

L = leaves, S = stem, P = whole plant.

hysterophorus and *Schinus molle* by OMM. Except for extracts of *P. hysterophorus*, the remaining extracts promoted conidial germination in the isolate from Veracruz when evaluated by EM (Lot 4). Similar effect was observed in the isolate from Guerrero when treated with *Erythrina americana*, *R. communis*, and *S. molle*. A negative percent germination (-7%) was obtained in the non-treated isolate from Guerrero and measured by EM after 14h. The extract of *Justicia*

Table 2. Evaluation of crude plant extracts by optical microscopy (OMM) and spectrophotometry method (EM) on conidia germination (%) of two isolates (Veracruz and Guerrero) of *Colletotrichum gloeosporioides*, after 14 and 18 h of incubation.

Scientific name	Veracruz				Guerrero			
	OMM		EM		OMM		EM	
	14 h	18 h	14 h	18 h	14 h	18 h	14 h	18 h
Lot 1								
Control	88b[z]	93b.	1a	8 a	72b	74b	16a	21b
Bougainvillea spectabilis	89b	93b	13b	16b	62a	64b	11a	15a
Annona muricata	86b	91b	29b	31b	67b	71b	18a	20b
Citrus aurantifolia	69a	81b	30b	31b	58a	67b	16a	17b
Citrus aurantium	74a	78a	41c	45c	44a	48a	28b	32c
Carica papaya	71a	83a	25b	28b	43a	55a	20a	22b
Lot 2								
Control	96b	96b	28b	36b	97b	100b	11a	22b
Persea americana	95b	95b	58d	64d	100b	100b	72c	77d
Ficus nitida	94b	96b	51c	51c	100b	100b	50b	55c
Psidium guajava	94b	96b	41c	57c	98b	99b	45b	59c
Cestrum nocturnum	1a	1a	1a	1a	0a	1a	5a	1a
Mangifera indica	81b	86b	18b	33b	100b	100b	7a	53c
Lot 3								
Control	100a	100a	29c	33c	97a	98a	25c	34b
Pithecellobium dulce	99a	100a	15b	21b	92a	98a	12b	21b
Mentha piperita	99a	100a	10b	14b	98a	99a	9a	16b
Piper auritum	100a	100a	13b	23b	96a	100a	14b	20b
Passiflora edulis	98a	100a	1a	8a	92a	97a	5a	9a
Cymbopogon citratus	100a	100a	36c	47c	98a	99a	29a	41c
Lot 4								
Control	81b	81b	6a	11a	76a	82b	-7a	8a
Erythrina americana	70b	86b	20b	37c	79a	80b	15b	26b
Ricinus communis	56a	78b	20b	40c	79a	87b	18b	31b
Azadirachta indica	73b	91b	19b	29b	77a	90b	6a	15a
Parthenium hysterophorus	64a	74a	1a	13a	73a	74a	2a	12a
Schinus molle	61a	69a	30c	56d	67a	75a	29c	47c
Lot 5								
Control	91b	92a	11b	22b	79b	83a	15b	22b
Piper aduncum	96b	99a	18b	29b	89b	99c	1a	12a
Brassica oleracea	94b	94a	40c	48c	86b	88b	3a	24b
Chenopodium ambrosioides	96b	97a	16b	27b	71a	88b	16b	34c
Justicia spicigera	48a	91a	0a	0a	74a	95b	1a	4a
Petroselinum sativum	95b	99a	39c	52c	67a	94b	1a	15b
Lot 6								
Control	96b	99b	21b	37b	96b	97b	17b	27b
Annona reticulata	97b	98b	5a	4a	95b	97b	1a	0a
Barkleyanthus salicifolius	97b	98b	26b	34b	95b	97b	8b	15b
Annona cherimola	2a	2a	2a	2a	2a	3a	4a	4a
Inga jinicuil	96b	98b	23b	30b	92b	96b	3a	7a
Diospyros ebenaster	97b	99b	2a	2a	91b	98b	1a	2a
Lot 7								
Control	100b	100b	19c	35c	83b	89b	3b	17c
Ocimum basilicum	100b	100b	2b	3b	83b	91b	-7a	-16a
Origanum majorana	39a	75a	1b	2b	69a	86a	1b	2b
Rosmarinus officinalis	96b	100b	-8a	-8a	85b	96b	-11a	-11a
Ruta chalepensis	100b	100b	32d	44d	84b	98b	10b	22c
Thymus vulgaris	96b	100b	2b	5b	86b	96b	-8a	-3a

Table 2. Continuation....

Scientific name	Veracruz				Guerrero			
	OMM		EM		OMM		EM	
	14 h	18 h	14 h	18 h	14 h	18 h	14 h	18 h
Lot 8								
Control	100a	100a	39c	48c	87a	94a	18c	32c
Allium schoenoprasum	100a	100a	2b	3a	92b	97b	-4a	-3a
Eucaliptus spp.	99a	100a	16b	24b	93b	98b	1b	7b
Punica granatum	99a	100a	2a	6a	94b	97b	-29a	-17a
Arctostaphylos polifolia	99a	100a	13b	18b	93b	96b	1b	3b
Nicotiana glauca	99a	100a	26c	36c	95b	98b	13c	33c

[z]Means followed by the same letter are not significantly different (P = 0.05) within lots, as determined by Kruskal Wallis (SNK). Average of six observations.

spicigera presented the best fungicidal effect (0%) after the given incubation times in the isolate from Veracruz. Other extracts that showed a good fungicidal effect over the isolate from Guerrero were *Piper aduncum*, *Brassica oleraceae*, and *Chenopodium ambrosioides* by EM (Lot 5). In Lot 6, determinations by OMM and EM showed that the best antifungal effect was when the two isolates were treated with extract of *Annona reticulata* (0-5%) (p = 0.05) at 14, and 18h. Similarly, extracts of *Annona cherimola* and *Diospyros ebenaster* had a good fungicidal effect as seen in the lowest germination of 2 to 5% (p = 0.05) in both isolates and determined by EM. In Lot 7, the lowest conidial germination determined by EM was obtained with extract of *Origanum majorana* in the isolate from Veracruz and Guerrero at 14 and 18 h. Negative conidial germination was shown in the isolate from Guerrero with extracts of *Ocimum basilicum*, *Rosmarinus officinalis*, and *Thymus vulgaris* by EM at both incubation periods. In Veracruz isolate treated with extracts of *Allium schoenoprasum*, *Eucaliptus* spp., *Punica granatum* and *Arctostaphylos polifolia* significantly (p = 0.05) lower germination than control treatment was observed by EM after 14 and 18 h, while in Guerrero isolate the best fungicidal extracts were *Eucaliptus* spp. and *A. polifolia*. Again negative values were observed with extracts of *A. schoenoprasum* and *P. granatum* (Lot 8). In our study, conidial germination varied according to type of isolate, extract applied, time of incubation and method evaluated. It is difficult to point out what of the above factors may have more effect on conidial survival however, in a previous study the same isolates of *C. gloeosporioides* showed differences in mycelial growth and germination associating these differences to the great genetic variability of this fungus (Hernández-Albíter *et al*. 2005). In general, the best antifungal effect was observed with plant extracts of *C. nocturnum* and *A. cherimola*. Extracts from various plant organs of *C. nocturnum* have shown to have high fungistatic activity over *Fusarium moniliforme* J. Sheld. (Bravo *et al*., 2000) and *C. gloeosporioides* (Grainge and Ahmed, 1988). The leaves of *C. nocturnum* have pharmacological significance in Chinese folk medicine and have been used for the treatment of burns and swellings (Xiao, 1989). Various glycosides such as 25R-spirost-5-ene2alfa, 3beta-diol pentaglycosides (nocturnoside A) (Ahmad *et al*., 1991), 25R-spirost-5en-3beta-ol tetraglycoside (nocturnoside B) (Ahmad *et al*., 1995), and phenolic glucosides (cesternosides A and B) (Sahai *et al*., 1994) were isolated from leaves of *C. nocturnum*, some of which showed cytotoxic activity against cultured tumor cells (Mimaki *et al*., 2001; 2002). In general, plants belonging to the Annonaceae family present cytotoxic, antitumoral, pesticide, and antibacterial activities (Bories *et al*., 1991; Rupprecht *et al*., 1990). In other studies, leaf extracts of *A. cherimola* inhibited spore germination of *C. gloeosporioides*, sporulation of *Rhizopus stolonifer* (Ehrenb.:Fr.) Vuill. and rot development of *Spondias purpurea* L. (red mombin) (Bautista-Baños *et al*., 2000a; b). Leaf cloroformic extracts shown antibacterial and antifungal activity against *Staphylococcus aureus* Rosenbach, *Mycobacterium phlei* Lehmann and Neumann, and *Candida albicans* (C.P. Robin) Berkhout (Márquez *et al*., 1999). Various acetogenins and alkaloid were isolated from the stems of *A. cherimola* (Chen *et al*., 1997a; b; 1998; 1999a; b; 2001).

CONCLUSIONS

Results of this investigation demonstrated the fungicidal potential of a range of crude plant extracts and present two main characters: a) their natural origin which means safer to people and the environment, and b) they can be considered as low risk for resistance development by pathogenic fungi. Natural plant-derived fungicides should provide a wide variety of compounds as alternatives to synthetic fungicides. It was not possible to relate results from spectrophotometry´s method and microscopy´s method. Interference in the measurement of absorbance values due to pigments, plant tissue, and crystal of the crude extracts and differences in the size of the germinating tube of the conidia as well, caused a high heterogeneity of mixture.

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