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In vitro control of anthracnose (*olletotrichum gloeosporioides*) isolated from *annona muricata* l. with vegetable extracts

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— Abstract—

Colletotrichum gloeosporioides is a disease of great importance in soursop cultivation since it can cause large production losses by being present in all the crop's phenological stages as an alternative to chemical synthesis products for its control. The in vitro effect of eight extracts in hydrolate form obtained from *Bougainvillea* spp., *Hibiscus sabdariffa* L., *Mangifera indica* L., *Carica papaya* L., *Pimenta dioica* L. and *Psidium guajava* L. were investigated using the poisoned media technique. In the first stage, the hydrolates were evaluated at 50% concentration V/V, those that achieved total inhibition of the pathogen were revaluated to calculate their minimum inhibitory concentration (MIC), the daily growth of the radial diameter of the pathogen as well as the number of total and germinated conidia. The results show that all the evaluated plants have compounds with fungistatic capacity on C. *gloeosporioides* when tested *in vitro*; in the first stage, P. *dioica* L., D. *ambrosioides* L., M. *indica* L., and *Bougainvillea* spp. inhibited the total development of the pathogen, while H. *sabdariffa* L. and P. *guajava* L. showed a minor inhibition in mycelial growth; however, they showed high antisporulant capacity (99.45% and 83.33% respectively); on the other hand, C. *papaya* showed low inhibition in both sporulation and mycelial growth. In the second stage, only P. *dioica* L. inhibited the total development of C. *gloeosporioides*, for which it achieved the lowest MIC with 40%, the other treatments failed to inhibit mycelial growth but all showed antisporulant capacity according to the comparison test Stocking by Tukey. The P. *dioica* L. hydrolate showed the lowest minimum inhibitory concentration with 40% (V/V), while for the hydrolates the D. *ambrosioides* L., *Bougainvillea* spp. (leaf, flower, and bract) and M. *indica* L., the minimum inhibitory concentration was 50% (V/V) on C. *gloeosporioides*.

Keywords:

Colletotrichum gloeosporioides; hydrolates; secondary metabolites; anthracnose; soursop; inhibition.

The soursop, *Annona muricata* L., is the most important species in the Annonaceae family, it is a crop with great economic potential, being demanded in agribusiness, the perfumery industry, and even pharmacology, taking advantage not only of the fruit but also the leaves and seeds (Vit, *et al*., 2014., León-Hernández, *et al*, 2019). Mexico has the climatic characteristics that allow it to be the country with the largest production area worldwide (National Institute for Forestry, Agricultural and Livestock Research - INIFAP, 2015; Reyes-Montero, *et al*., 2018). However, the scarce agronomic research around the problems in the crop production represent an obstacle to the development of producers, mainly small and those who carry out organic production. Among the productive problems faced by this crop, is anthracnose, caused by the fungus *Colletotrichum gloeosporioides*, a phytosanitary problem that causes large economic losses (Anaya-Dyck, *et al*., 2021).

C. *gloeosporioides* is characterized by a remarkable infective capacity, causing considerable losses in both crop production and postharvest, (Landero-Valenzuela, *et al*., 2016; Betancourt, 2019), infecting seedlings and adult plants by attacking flowers, branches, stems, leaves and fruits (Sáyago and Álvarez, 2018), causing dark brown to black lesions, falling of flowers, fruits and leaves (Hernández and López, 2018).

The traditional control of this disease consists of practices of cultural work as well as spraying of chemical synthesis products. However, these products have been used irrationally and, in many cases, erratically, which has consequently generated health and human health problems; in addition to a serious ecological imbalance favoring the emergence of pests and diseases more aggressive and resistant to certain substances that are traditionally used for their control (Gordillo, 2019).

Due to the above, agriculture is often mentioned as one of the factors that have contributed greatly to environmental pollution and climate change, so it is necessary to change the current agricultural production model to a sustainable production model that ensures the ecological harmony of agroecosystems.

On the other hand, both the production and consumption of soursop have increased in recent years at a national level (Terán-Erazo, *et al*., 2019), which presents a need for research aimed at generating strategies that allow the incorporation of soursop cultivation into a sustainable system. An interesting alternative to contribute to this is using plant extracts to replace synthetic products for phytosanitary problems management.

Previous research has shown the advantages and success of using plant extracts on different microorganisms and even insects. In this framework, extracts of *Bougainvillea* spp. have been tested on *Botrytis cinerea* (Santiago,

et al., 2019) and C. *gloeosporioides* (Hernández, 2004), obtaining inhibitory results. On the other hand, D. *ambrosioides* L. has been shown to have a positive effect on various phytopathogenic fungi (Cabrera, *et al*., 2016; Montes-Belmont and Carvajal, 1997; Rivera-Castañeda, *et al*., 2001; Vasquez, *et al*., 2014;) and even on *Spodoptera frugiperda* larvae (Chávez, 2019).

Another plant species that have reported antifungal properties is *H. sabdariffa* L., whose extract achieved a total inhibitory effect against *Alternaria solani* (Goussou, *et al*., 2010). On the other hand, extracts of *P. dioica* L. have been successfully tested on different fungal pathogens (Aguilar, *et al*., 2019; Arcos-Méndez, *et al*., 2019; Ramírez-González, *et al*., 2007). The *C. papaya* L. seed insecticidal (Figueroa, *et al*., 2011; Franco-Archundia, 2006) and fungicide capacity has been previously reported (Ramírez-González, *et al*., 2007). Likewise, authors report inhibition when evaluating extracts of *P. guajava* L. in vitro versus *C. gloeosporioides* (Baños-Guevara, 2003), while *M. indica* L. stands out for having an antimicrobial effect against bacteria of health interest (Guerra & Román, 2016).

Plant extracts, being products obtained from renewable materials, have the advantage of being able to degrade quickly, be harmless to the environment, and be selective with pests and diseases (Ibáñez & Zoppolo, 2008; Figueroa, *et al*., 2019), in addition to being economical, reducing negative impacts on the ecological balance and contributing to the development of small producers.

Considering the biological diversity of Mexico, it is interesting to explore the potential of plant extracts in the control of diseases such as anthracnose. That is why the objective of this research is to evaluate the potential use of eight botanical extracts in the control of the pathogen's growth that causes anthracnose in soursop (*Colletotrichum gloeosporioides*), taking into account the importance of the culture and the relevance of the disease in terms of damage caused, and in this way, Contribute to the resolution of one of the most important phytosanitary problems of soursop cultivation, acting within the framework of sustainable agriculture.

METHODOLOGY

The experimental process was developed in the Agrotecnologías de la Agencia Universitaria para el Desarrollo (AUDES) Cacao-Chocolate of the Universidad Autónoma de Chiapas (UNACH). The plants used for the preparation of the extracts were: *Bougainvillea* spp. (leaves, bracts, and flowers), *Dysphania ambrosioides* L. (stem and leaves), *Mangifera indica* L. (leaves), *Carica papaya* L. (seeds) fresh and dry, *Hibiscus sabdariffa* L. (flower), *Psidium guajava* L. (leaves) and *Pimenta dioica* L. (fruit).

C. gloeosporioides was previously isolated from a soursop fruit of a crop located in the municipality of Tecpatán, Chiapas; its multiplication was carried out in a PDA medium using a punch and allowed to grow for 12 days.

To obtain the hydrolates, the methodology proposed by Ramírez (2013) was followed, using the method described as hydrolates by distillation, as a solvent, the ethyl alcohol in a 10:1 ratio, as reported by Tamayo (2016), was used as the water mixture. The hydrolates obtained were stored in sterile Erlenmeyer flasks and refrigerated at 4°C for later use.

Evaluation of hydrolats at 50% concentration

The hydrolates were evaluated at 50% concentration in a PDA medium, using the poisoned media technique, a completely randomized design was established with ten treatments and three repetitions each, eight treatments corresponding to the hydrolates, a chemical control (i.a. Chlorothalonil) and an absolute control (PDA). Inoculation of the pathogen was performed using a punch. The inhibitory effect was quantified by measuring the growth of the pathogen mycelial diameter every 24 hours for 12 days. Likewise, the production of total and germinated spores was quantified using the Neubauer chamber following the methodology described by Gilchrist, *et al*. (2005). The data obtained were used to calculate inhibition percentages on growth and sporulation.

Determination of minimum inhibitory concentration (MIC)

The minimum inhibitory concentration (MIC) of the treatments that in the first trial managed to inhibit the pathogen was determined, for this, concentrations of 40%, 30%, 20%, and 10% were evaluated, following the same methodology described above.

Statistical analysis

To determine the effects of the treatments studied, a variance analysis (ANOVA) was performed, and the 5% Tukey Mean Comparison Test was applied, using SPSS version 17.0 software for Windows.

RESULTS

Hydrolates effect at 50% concentration

Bougainvillea spp., *D. ambrosioides* L., *M. indica* L., and *P. dioica* L. hydrolates, inhibited the development of the pathogen by 100%, surpassing even the result obtained by the chemical control, which inhibited mycelial growth by 75.33%. The statistical analysis shows that these results are statistically different from each other; in the case of the sporulation variable, the chemical control inhibited by 98.53%, a result statistically identical to the treatments results mentioned above. On the other hand, hydrolates based on *H. sabdariffa* L. and *P. guajava* L. showed high antisporulant capacity with 99.45 and 83.33%, respectively, results statistically identical to those achieved by the chemical control and treatments that completely inhibited the pathogen; however, its inhibitory capacity on mycelial growth of *C. gloeosporioides* was reduced. In the case of *C. papaya* L., the results report a reduced capacity in both growth inhibition and sporulation, inhibiting below 50% in both cases (Table 1).

Table 1
Mycelial growth and sporulation of C. gloeosporioids by 50% concentrated hydrolate effect

	Mycelial growth			Conidia concentration		
Treatment	Growth (mm)	Tukey HSD[a]	Inhibition (%)	Conidias/mL *10^{11}	Tukey HSD[a]	Inhibition (%)
***Bougainvillea* spp. (leaf)**	0	A	100	0	A	100
***Bougainvillea* spp. (flower and bract)**	0	A	100	0	A	100
***D. ambrosioides* L.**	0	A	100	0	A	100
***M. indica* L.**	0	A	100	0	A	100
***P. dioica* L.**	0	A	100	0	A	100
***H. Sabdariffa* L.**	26.67	C	46.67	2.93	A	99.45
***C. papaya* L.**	47.67	D	4.67	285.33	B	46.89
***P. guajava* L.**	48.33	D	3.33	78.81	A	83.33
Chemical control	12.33	B	75.33	7.91	A	98.53
Absolut control	50	D	0	537.27	C	0

*Averages with the same letter in the same column show no statistically significant differences in the Tukey test ($p<0.05$).

Source: Own elaboration

Determination of minimum inhibitory concentration (MIC)

The treatments corresponding to *Bougainvillea* spp., *D. ambrosioides* L., *M. indica* L., and *P. dioica* L. that at a 50% concentration completely inhibited the pathogen, were evaluated at this stage in different concentrations. Only the hydrolate based on *P. dioica* L. affected the growth of the pathogen, achieving a total inhibition of the same at a 40% concentration, showing an increase in the concentration of conidia by decreasing the concentration of the hydrolate in the culture medium, similar behavior observed in all treatments (Table 2). The statistical analysis showed significant differences between the treatments corresponding to *P. dioica* L., so the MIC for this treatment was established at 40%.

On the other hand, all treatments showed antisporulant capacity, highlighting those corresponding to *Bougainvillea* spp. (bracts) at 40 and 30%, *Bougainvillea* spp. (leaves) at 40, 30, and 20%, *D. ambrosioides* L. in all concentrations, *M. indica* L. at 40, 30, and 20%, and *P. dioica* L. at 30% indicating an inhibition greater than 70%. It should be noted that all concentrations of *D. ambrosioides* were able to inhibit pathogen sporulation above 90%. Only *Bougainvillea* spp. (bracts and leaves) and *P. dioica* L. at 10% showed less than 50% inhibition. The analysis of variance showed differences between the treatments, and the comparison test of means by Tukey showed that no treatment is statistically identical to the result of the Absolute Control, so they all have antisporulant capacity (Table 2). It should be noted that there were no germinated conidia present for any treatment in the trials.

Table 2

Mycelial growth and sporulation of C. gloeosporioids at 40%, 30%, 20%, and 10% concentrated hydrolate effect

Treatment	Mycelial growth			Conidia concentration		
	Growth (mm)	Tukey HSD[a]	Inhibition (%)	Conidias/mL *10^{11}	Tukey HSD[a]	Inhibition (%)
***Bougainvillea* spp.**	50	D	0	12.77	F	86.36
***Bougainvillea* spp. (flower and bracts)-30%**	50	D	0	23.68	I	74.70
***Bougainvillea* spp. (flower and bracts)-20%**	50	D	0	40.01	L	57.25
***Bougainvillea* spp. (flower and bracts)-10%**	50	D	0	90.30	N	3.52
***Bougainvillea* spp. (leaf)-40%**	50	D	0	9.68	R	89.66
***Bougainvillea* spp. (leaf)-30%**	50	D	0	17.44	G	81.37
***Bougainvillea* spp. (leaf)-20%**	50	D	0	27.94	J	70.15
***Bougainvillea* spp. (leaf)-10%**	50	D	0	50.27	M	46.29
***D. ambrosioides L.*-40%**	50	D	0	2.27	AB	97.57
***D. ambrosioides L.*-30%**	50	D	0	4.23	BC	95.48
***D. ambrosioides L.*-20%**	50	D	0	5.95	CD	93.64
***D. ambrosioides L.*-10%**	50	D	0	8.02	DE	91.43
***M. indica L.*-40%**	50	D	0	3.15	B	96.63
***M. indica L.*-30%**	50	D	0	9.43	E	89.92
***M. indica L.*-20%**	50	D	0	21.84	I	76.66
***M. indica L.*-10%**	50	D	0	37.84	L	59.57
***P. dioica L.*-40%**	0	A	100	0	A	100
***P. dioica L.*-30%**	10	B	80	17.93	H	80.84
***P. dioica L.*-20%**	30	C	40	28.81	JK	69.22
***P. dioica L.*-10%**	50	D	0	50.52	M	46.06
Absolut control	50	D	0	93.59	N	0

*Averages with the same letter in the same column show no statistically significant differences in the Tukey test ($p<0.05$).

Source: Own elaboration

DISCUSSION

The hydrolate of *P. guajava* L., allowed the greatest mycelial growth, in contrast to these results, other authors report inhibition versus *C. lindemuthianum*; however, they do not report inhibition for *M. fructicola*, A. pisi, and *P. parasítica* (Villanueva, *et al*., 2012). Regarding these differences in the studies, several authors mention that fungistatic activity differs between the different forms of extraction, the species of the plant, and the pathogen evaluated (Hernández, *et al*., 2007; Sánchez, 2019). On the other hand, this treatment showed significant inhibition on the concentration of conidia, evidencing anti-sporulant qualities, this information coincides with that reported by Bravo, *et al*., (2000), who evaluated powders of this plant obtaining an anti-sporulant effect against *Fusarium moniliforme*. This capacity may be due to the secondary metabolites present in its structure, such as phenols, flavonoids, triterpenes, and saponins, among others, which have been reported with antifungal capacity (Rodríguez, *et al*., 2013; Mas, *et al*., 2017).

On the other hand, *H. sabdariffa* L. showed high antisporulant capacity; information that adds to that reported by Goussous, *et al*., (2010), who report total inhibition on A*lternaria solani*, using raw extracts from this plant, attributing these results to the presence of a polyphenol called protocatechuic acid. Similarly, the antimicrobial capacity of different extracts of this plant against bacteria such as *Salmonella enteritidis* and *Excherichia coli* has been previously tested (Castillo, 2018), thus demonstrating the antimicrobial potential of the plant.

The results obtained for the treatment with C. papaya L. seed hydrolate are partially like those obtained by Bautista-Baños, *et al*., (2003), who tested *C. papaya* L. seed extract on *C. gloeosporioides* papaya isolate and obtained a zero inhibition both in sporulation and in mycelium growth; however, there are methodological differences in the preparation of the extracts evaluated in the different studies.

The results obtained using the hydrolate of *P. dioica* L. are added to those obtained by other authors such as Ramírez-González, *et al*. (2007), who report total inhibition of *Phytophthora palmivorausing* 50% concentration of *P. dioica* hydrolate. Duarte, in 2019, reports the use of an extract of *P. dioica* obtained by microwave at 50% concentration, obtaining total inhibition against *C. gloeosporioides* and *A. alternata*. Pepper is recognized for its remarkable antifungal activity due to the secondary metabolites present in its structure, such as essential oils, tannins, flavonoids, phenols, and terpenes (Álvarez, *et al*., 2010; Velázquez-Silva, *et al*., 2019).

In the case of *D. ambrosioides* L., the results of this study improve those obtained by authors such as Cabrera, *et al*. (2016), who report 79% inhibition against *C. gloeosporioides* in tests *in vitro* using ethanolic extracts. The

antifungal capacity of epazote, demonstrated in this study, is added to the many reported qualities of this plant, such as pesticide against lepidoptera and beetle (Chávez, 2019), amebicide, analgesic, among others (López, 2020). Qualities that are attributed to the different metabolites reported in their structure, such as essential oil, phenols, flavonoids, and saponins, among others (Chávez, 2019).

In the case of *Bougainvillea* spp., the results achieved in this study reveal antifungal and antisporulant capacity, both for the leaf and the bracts. Other authors report having obtained significant inhibition on the germination of spores of *C. gloeosporioides* (Hernández, *et al*., 2004), in addition to inhibition against *Botritys cinerea* in blueberry fruits (Santiago, *et al*., 2019). The uses of this plant in both agriculture and traditional medicine have been previously reported (Edwin, *et al*., 2007; Galindo, *et al*., 2009), likewise, different compounds have been found both in leaves and in the bracts responsible for antifungal activity, such as the low molecular weight proteins called defensins (Hernández, 2004), in addition to flavonoids, tannins, alkaloids, and saponins, which perform defense functions in plants (Edwin, *et al*., 2007).

The results obtained in this study verify the antifungal and antisporulant capacity of the hydrolate of *M. indica* L., thus increasing knowledge about the properties of this plant, contrasting with previous investigations that have reported low inhibition in the germination of spores of *C. gloeosporioides*, using *indica* L. leave extracts, (Hernández-Altíber, *et al*., 2006). On the other hand, the antimicrobial capacity of the plant has been reported against bacteria of health interest, such as *Escherichia coli*, *Staphylococcuss aureus*, *Listeria monocytogenes* (Ortiz, 2015), *Pseudomona aeuroginosa, Salmonella Typhimurium, Enterococcus Faecalis* (Carrillo-Tomalá, *et al*., 2019), among others. These properties present in the mango leaves extract are attributed to their bioactive compounds such as polyphenols, flavonoids, and gallic tannins, which in addition to being antimicrobial, are antiviral, anti-inflammatory, antioxidants, etc., (Carrillo-Tomalá, *et al*., 2019; Ortiz, 2015).

The results show that although not all treatments managed to inhibit mycelial growth, they do possess the ability to inhibit the production of spores of *C. gloeosporioides*, since when compared to the absolute control, a reduction of these structures is noticeable; conidia are the main source of the inoculum for the dissemination and development of the disease (Díaz-Medina, *et al*., 2019) so a reduction in the number of these structures would substantially reduce the pathogenicity of the fungus and, consequently, its ability to initiate an infectious cycle (Valdés, *et al*., 2017). The inhibition of mycelial growth produced by hydrolates in the present work may be because naturally occurring compounds cause irreversible damage to the cell structure, affecting the physiology of the fungus. Phenolic compounds affect the

active sites of enzymes and cell metabolism by reducing the growth rate of the pathogen (D´Luis, 2018). In previous studies, it is reported that plant extracts can cause alterations in the structure and shape of pathogens; Duarte (2019) reports having observed dehydration in conidia of *C. gloeosporioides* treated with extract of *P. dioica*. On the other hand, alkaloids are related to the inhibition of protein synthesis, induction of apoptosis, and inhibition of carbohydrate metabolism enzymes (Duarte, *et al*., 2021, González-Chavarro, *et al*., 2020); however, these properties, present in the extracts, although attributed to their active compounds, highlight the fact of the synergy that exists between all the components of the extract, since the effect they achieve is not due to their individual action but to several reactions that act in a certain concentration and proportion (D´Luis, 2018; Hernández, 2019, Ramírez-González, *et al*., 2016).

CONCLUSIONS

Hydrolates of *P. dioica* L., *M. indica* L., *D. ambrosioides* L., *Bougainvillea* spp. (leaf, flower, and bract), *P. guajava* L e *Hibiscus sabdariffa* L., presented an *in vitro* inhibitory effect on the growth and development of *Colletotrichum gloeosporioides* isolated from *Annona muricata* L.

The hydrolate of *P. dioica* L. showed the lowest minimum inhibitory concentration with 40% (V/V), while for the hydrolates based on *D. ambrosioides* L., *Bougainvillea* spp. (leaf, flower, and bract) and *M. indica* L., the minimum inhibitory concentration was 50% (V/V).

The hydrolates based on *H. sabdariffa* L and *P. guajava* L. showed a high antisporulant capacity. The form of extraction used to obtain hydrolates from the plants tested was shown to be an effective way of controlling the development of phytopathogenic fungi at the laboratory level.

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