

Fungal Extracts as Biocontrol of Growth, Biofilm Formation, and Motility of *Xanthomonas citri* subsp. *citri*

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Abstract: For the present work, *Metarhizium rileyi*, a common entomopathogenic fungus, was grown in batch conditions in the absence and presence of its host, *Spodoptera frugiperda*, to obtain secondary metabolites with potential antimicrobial effect. The extraction of secondary metabolites was carried out by using two solvent systems for the biomass (ethyl acetate and methanol), while secondary metabolites present in the supernatant were extracted by using ethyl acetate as extraction solvent. These extracts were evaluated for their inhibitory effect on the growth, biofilm formation, and motility of three *Xanthomonas citri* subsp. *citri* strains (Xcc20, Xcc29, and Xcc42). The *in vivo* effect of these extracts to prevent the development of cankers on grapefruit leaves was also evaluated.

M. rileyi biomass ethyl acetate extracts in the presence and absence of its host had a strong inhibitory effect on Xcc strains growth. On the other hand, the presence of *S. frugiperda* as an elicitor significantly increased *M. rileyi*'s inhibitory capacity to form a biofilm. A different behavior was observed for the copper resistant strain, Xcc42, for its biofilm formation and swimming capacity since the most active samples were the supernatant extracts in the presence and absence of *S. frugiperda*. Our results suggest that the most important factor for *in vivo* canker development is the inhibition of Xcc's motility since all the extracts that inhibited canker development *in vivo* were also able to inhibit Xcc20's motility *in vitro*. The fact that the extracts can prevent cankers development *in vivo* indicates they are promising sources of metabolites to prevent the development of this citrus disease.

Keywords: Citrus cankers, *Metarhizium rileyi*, *Spodoptera frugiperda*, *Xanthomonas citri*.

1. INTRODUCTION

Citrus represents an important economic resource for Argentina, with more than 355,000 Tons exported in 2017; this value positions the country as the eight most important citrus producers of the world [1].

Citrus canker is one of the most important diseases that affect several citrus species cultivated in tropical and sub-tropical regions of the world. It is caused by *Xanthomonas citri* subsp. *citri* (ex Hasse) (Xcc), a Gram (-) plant-pathogen bacteria that infects seedlings and young susceptible citrus trees, causing erumpent necrotic lesions on leaves, branches, sprouts, and fruits [2]. Damage to mature tissues caused by biotic or abiotic factors facilitates infection by Xcc and the disease dispersal [3]. The principal dispersion agent is windy rainfalls and splashes. As a result, tropical storms and hurricanes can disperse the disease up to 53 km [4].

X. citri pv. *citri* (Xcc) persists on the environment as an epiphyte on the host and non-host plants, and as a

saprophyte on the soil or ground cover. Winter lesions are the principal source of inoculum for the next season outburst of the disease [5]. Once Xcc colonizes its host plant, it multiplies in the intracellular space of the infected tissue or colonizes the xylem vessels to spread within the plant [6].

Much effort is put into preventing the infection of healthy plants and the development of cankers caused by Xcc, given that, depending on the severity of the contamination, fruit's marketability and harvest losses can be substantial. International regulations demand a post-harvest treatment that includes sanitization of fruits and their preventive immersion in chlorine to reduce the microbial load and the risk of dispersion. Nonetheless, this measure has shown to be ineffective since analysis has detected Xcc on sanitized fresh fruits [4].

Common preventive measures on the field include isolation of Xcc free specimens by placing windbreaks around the healthy trees or by spraying the plants with copper-containing bactericides like mancozeb [3]. However, mancozeb has numerous adverse effects on human health after the acute and chronic exposition, such as cancer, reproductivity problems, organic toxicity, etc. [7]. Its products of degradation also have a

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negative effect on animals and the environment due to accumulation in groundwaters and soils [8]. Moreover, the environmental effect of copper-based pesticides is well documented [9]. New efforts are seeking for natural and non-toxic antimicrobials to prevent and control Xcc's cankers in endemic regions around the world since strains resistant to copper have already been reported [10].

Xcc pathogenicity is mediated by some virulence factor essential for the bacteria adherence to the plant surface, as well as to invade the intercellular space, acquire nutrients, and counteract the host defense response [6]. For the present work, grapefruit leaves were infected with the acquired strains, and the pathogenic bacteria were re-isolated for the subsequent experiments.

One of the main strategies of Xcc to colonize and infect the citrus involved the biofilm formation. This is a complex process that involves three main stages (attachment to the surface, proliferation/formation of biofilm, and dispersal), and requires the expression of numerous virulence factors, as well as the cooperative work mediated by *Quorum sensing* of all the bacteria attached to a biotic or abiotic surface [11]. Inhibiting biofilm synthesis could be achieved by blocking mechanisms involved in these three fundamental stages and bacteria communication through *Quorum sensing*.

Entomopathogenic fungi have been used as a biological control against many insect pests of important crops as a safe alternative to toxic and recalcitrant commercial pesticides [12]. These fungi produce a great array of molecules that range from proteins and enzymes that allow them to colonize and infect their host, and secondary metabolites whose function is aimed to facilitate the adhesion and to counteract the immunological defense of their host [13].

Metarhizium rileyi is an entomopathogenic fungus that infects a wide variety of insect pests with great economic importance [14]. The fungi also synthesize antimicrobials that target symbiotic bacteria that inhabit the insect surface and participate in their host defensive system [15]. This capacity to produce antimicrobial molecules along with their high production of biomass indicates that they could also be used as natural antimicrobial biopesticides to prevent Xcc cankers. For the present work, we evaluated the potential use of secondary metabolites of *M. rileyi* as antimicrobial agents against Xcc.

2. MATERIAL AND METHODS

2.1. *Xanthomonas citri* subsp. *citri* Strains

Xanthomonas citri pv. *citri* (Xcc) was provided by the Estación Experimental Agroindustrial Obispo Colombres (EEAOC), Tucumán, Argentina. The strains employed for this work were obtained from citrus cankers on the different parts of citrus that were cultivated in San Andrés and Las Talitas (Tucumán, Argentina) (Table 1). The strain Xcc42 was isolated from cankers growing on lemon plants previously treated with copper bactericides.

Table 1: *Xanthomonas citri* subsp. *citri* Strains

Strain number	Location	Plant source
Xcc20	San Andrés	Lemon leaf
Xcc29	EEAOC	Grapefruit
Xcc42	San Andrés*	Lemon

*This strain was isolated from plants previously treated with copper.

Strains preserved in phosphate-buffered saline (PBS) were activated by growing in nutrient agar for 48 h at 28°C, under aerobic conditions. Afterwards, single colonies were grown in Luria-Bertani medium (LB) at 28°C for 48 h under aerobic conditions and without shaking.

2.2. Pathogenesis Test

Duncan grapefruits (*Citrus paradise*) were used for *in vivo* assays because of its major sensibility to Xcc. Duncan grapefruits were grown in a greenhouse at 22-25°C with a photoperiod of 16 h light hours. When seedlings reached 70 cm of height, their leaves were selected and numbered from the apex (most sensible to infections). Leaves were infected by inoculating 5 mL of each Xcc strain suspension (Xcc in sterilized distilled water, DO_{560nm} = 0.1) using a needle-less syringe. The infected plants were incubated for 7 days in a greenhouse at 20-25°C with a photoperiod of 16 h light hours.

2.3. Re-Isolation

Symptomatic leaves were collected and cleaned with ethanol 96°. Afterwards, cankers were cut using a sterile scalpel (portions of 3x3 mm) and were smashed with two drops of sterile water to resuspend the cankers' bacteria. After 15 min, an aliquot was transferred to nutrient agar, and it was incubated at 28°C for 48 h under aerobic conditions. To isolate pure

colonies, bacteria were inoculated in a plate with nutrient agar by streaking and were incubated at 28°C for 48 h.

2.4. Preparation of the Fungal Extracts

2.4.1. Fungal Strain

For the present work, an ARSEF 4094 strain of *Metarhizium rileyi* (Farlow Samson) was used. It was isolated from *Spodoptera frugiperda* (Lepidoptera: Noctuidae) in Bahía, Brazil, in 1985. The strain was provided by the ARS Collection of Entomopathogenic Fungal Cultures (ARSEF, USA).

2.4.2. Activation of *Metarhizium rileyi*

The strain, obtained as lyophilized spores, was activated by injecting 1 mL of distilled water and was left for 15-30 min to reconstitute the cellular components. Afterwards, 500 µL of the suspension was inoculated in Sabouraud maltose agar supplemented with yeast extract 1% (SAMY) and were incubated at 25°C for 10 days. These conditions induced a micellar growing of the fungi. *M. rileyi* was kept under this condition for another 15-20 days to obtain spores.

2.4.3. Preparation of the Spores Inoculate

The sporulated cultures grown in SAMY medium were washed using Sabouraud maltose liquid medium supplemented with yeast extract 1% (SMY) to reach an absorbance of 0.9-1.0 at 560 nm. This absorbance corresponded to a concentration of 10^8 spores/mL. Spores were counted using a Neubauer chamber.

2.4.4. Batch Culture of *Metarhizium rileyi*

SMY was used as a base to prepare the following growing mediums:

Medium F (fungus) (M. rileyi control): 300 mL of medium SMY were inoculated with a spore suspension of *M. rileyi* ($DO_{560nm} = 0.9$) for a final concentration of 3% (v/v).

Medium FI (fungus + insect) (M. rileyi-S. frugiperda): 300 mL of medium SMY were inoculated with a spore suspension of *M. rileyi* ($DO_{560nm} = 0.9$) for a final concentration of 3% (v/v). This medium was also supplemented with sterilized fragments of *S. frugiperda* 1% (w/v).

Medium I (insect) (S. frugiperda control): 300 mL of medium SMY were inoculated with 1% (w/v) sterilized fragment of *S. frugiperda*.

Each medium was incubated under constant shaking (180 rpm) at 27°C for 15 days. The experiments were carried out by triplicate.

2.5. Extraction of Active Principles

The culture medium was filtered using Whatman N°1 filter papers to separate the biomass from the liquid medium. Supernatants obtained were subjected to two consecutive extractions using equal volume ethyl acetate (50% v/v), while a third extraction was carried out using ethyl acetate 25% v/v. The active principles present in the biomass were extracted in three cycles for 48 h by using ethyl acetate, and a fourth extraction was carried out using methanol to obtain more polar metabolites.

Anhydrous sodium sulfate was added to all the extracts obtained to eliminate water remains. Afterwards, extracts were filtered using Whatman N°1 filter papers and evaporated *in vacuo* at a maximum temperature of 45°C. For the biological assays, the obtained extracts were resuspended in DMSO:H₂O (1:1) to obtain stock solutions of 2 mg/mL.

2.6. Analysis of Chemical Complexity by Thin-Layer Chromatography

Each extract was spotted onto a TLC plate (silica gel, Kieselgel G60 F254 0.2 mm). Afterwards, plates were developed using chloroform: ethyl acetate (7:3 v/v) as a mobile phase. Plates were dried and visualized under UV light of 254 and 365 nm (UV lamp model 5L-58 Mineralight Lamp). To evaluate the presence of sterols, the developed plates were also sprayed with Godin reagent (1 vol 1% vanillin in ethanol +1 vol 3% perchloric acid in water) and 30% sulfuric acid and were heated at 100°C until bands were visible [16].

2.7. Antimicrobial Assay

2.7.1. Inhibition of the Bacterial Growth

The biomass and supernatant extracts' effect on the growth of the strains Xcc20, Xcc29 and Xcc42 were determined spectrophotometrically using the microdilution method, according to Sandasi *et al.* (2012) [17].

After incubation for 24 h in Luria-Bertani medium, strains were diluted using the fresh medium for a 4% (v/v) final concentration. An aliquot of the cells suspension (180 µL, 1×10^6 cells/mL) and 20 µL of each extract 1 mg/mL (100 µg/mL as a final concentration in

the well) were transferred to microplate wells and were incubated for 48 h at 28 °C. The bacteria's growth was determined spectrophotometrically at OD_{560 nm} using a microplate reader (Power Wave XS2, Bio Tek, Vermont, USA). All the assays were done by sextuplicate in two series of experiments. An equivalent volume of DMSO:H₂O (1:1) was added instead of the extracts as growth control, while ciprofloxacin (5 µg/mL) was used as a positive control. The maximum level of DMSO to which the cells were exposed was 2.5%.

2.7.2. Inhibition of Biofilm Formation

The inhibition of the biofilm formation was determined spectrophotometrically using the crystal violet technique [18] with some modifications [19].

Xcc strains prepared as described in the previous paragraph were incubated for 48 h at 28 °C in the presence of the extracts (100 µg/mL final concentration). Afterwards, the supernatant was eliminated, and 20 µL of crystal violet 0.1% (w/v) were added to each well and was left to react for 30 min at room temperature. For 100% biofilm formation control, DMSO:H₂O (1:1) was added instead of the extracts, while ciprofloxacin (5 µg/mL) was used as a positive control.

The crystal violet solution was eliminated by inversion of the plates and washing with distilled water. The biofilm adhered to the plates was dissolved using absolute ethanol. The absorbance was determined at 540 nm using a microplate reader (Power Wave XS2 Biotek, Vermont, USA). All the assays were done by sextuplicate in two series of experiments.

2.7.3. Inhibition of Motility

This assay was carried out using the Luria-Bertani medium supplemented with agar 0.5% and 0.25%, according to Ha *et al.* (2014) [20] with slight modifications. Both media were added to a Petri dish in two layers, with the medium supplemented with 0.5% agar forming the underlayer and the medium with 0.25% agar as the upper layer. After solidification, 3 µL of each extract was spotted in the central part of the plates and were dried for 1 h. Afterwards, 5 µL of each Xcc strain (DO_{600nm} =0.8) was added to the extracts' spots and was dried for 1 h. Plates were incubated for 48 h at 28°C. The fungal development can be observed as a circular colony at the point of inoculation of a more intense color, and swimming is observed as a lighter halo around the colony. The motility diameter of the bacteria from the inoculation point was measured with the ImageJ program and compared with the control

(DMSO: H₂O) to calculate the percentages of migration inhibition.

2.8. In Vivo Assay

2.8.1. Inhibition of Cankers Development

Duncan grapefruit leaves were numbered starting from the apex and were cut using a sterile needle. Afterwards, each extract was added using clean cotton, and they were left to dry for 1 h. A suspension of the strain Xcc20 (2.5 µL, DO_{560 nm} =0.8) was added to the underside of the leaves using clean cotton. The infected plants were incubated for 15 days in a greenhouse at 20-25°C, with a photoperiod of 16 light hours.

3. RESULTS AND DISCUSSION

3.1. Re-Isolation from Infected Leaves

Xcc strains grow on nutritive agar forming yellow colonies of smooth edges and elastic texture. In a bid to verify their virulence, these colonies were used to infect leaves of Duncan grapefruit to obtain cankers, and the active strains were re-isolated. This allowed us to recover three pathogenic Xcc strains: Xcc20, Xcc29, and Xcc42. These strains were grown in Luria-Bertani medium to evaluate their capacity to form biofilm after 48 h of incubation. It was observed that all the strains produced biofilm.

3.2. Fungi Extracts

3.2.1. Activation and Growth of *Metarhizium rileyi*

M. rileyi is an imperfect dimorphic fungus that initially has a yeast-like growth and evolves into a filamentous development after a few days [21]. Sporulation is characterized by a variation of the mycelium's color, which goes from cottony white to an intense green during sporulation. Since this fungus has high nutritional requirements, they were grown in Sabouraud-maltose added with 1% yeast extract (SMY) before mass production in batch conditions.

Metarhizium rileyi (Farlow) Samson (ARSEF 1972) showed a good growth after 10 days of incubation at 25°C on solid medium, forming a cottony white mycelium, that was hard to separate from the agar medium. After 15-20 days of incubation, spores were visible as the white mycelium turned green.

Afterwards, the fungus was grown in a batch medium in the presence and absence of *Spodoptera frugiperda* fragments for 15 days. Under rotary shaking,

M. rileyi exhibited a tightly packed micellar growth with a tendency to form spheres, and conidial production was absent. The amount of biomass obtained showed a 38% increase when the medium was supplemented with *S. frugiperda*'s fragments (144 g/L) compared to the non-supplemented medium (104 g/L). It is known that some entomopathogenic fungi require an influx of nutrients derived from the insect's cuticula to germinate. Fungi's hydrolytic enzymes also release compounds from the insect's surface that act as specific signals that induce germination, the formation of the germ tube, etc. [22]. This specific behavior of entomopathogenic fungi in response to their interaction with their hosts might explain the higher yield obtained for the medium supplemented with *S. frugiperda* fragments.

3.2.2. Extraction of Secondary Metabolites

Entomopathogenic fungi synthesize a great array of secondary metabolites, mainly hydrolytic enzymes and low polar compounds, that help them to overcome the host's physical and immunological defenses. Their functions range from the degradation of the insect's cuticula to antibiosis and antifeedant properties, inhibition of the host's growth and immunological system, among others [23]. Gene clusters produce some of these metabolites constitutively, while others are specifically produced in response to signals associated with fungi interaction with their host.

Metarhizium rileyi belongs to Hypocreales order, which groups a great number of entomopathogenic fungi. Fungi belonging to this order synthesize a group of secondary metabolites that seems to be characteristic of Hypocreales' species (polyketides, terpenoids, non-ribosomal peptides, etc.) [23]. For this work, two solvent systems of different polarities (methanol and ethyl acetate) were used to extract secondary metabolites produced by *M. rileyi* that are released to the extracellular medium or attached to its membrane. Six extracts were obtained from the biomass, and three were obtained from the supernatant fermented medium (Figure 1).

3.2.3. Qualitative Analysis of the Extracts

Each extract was spotted onto a TLC plate to observe possible differences in *M. rileyi*'s chemical profile in the presence and absence of *S. frugiperda* fragments. Godin reagent was used as a revealing agent. This reagent reacts with polyols, phenols, and ketones, giving visual spots whose color range from yellow, brown and green for flavonoids, to different shades of blue, gray and violet for terpenoids [24,25].

Figure 2 shows the TLC profiles of the biomass and supernatant ethyl acetate extracts, and the methanolic biomass extracts, which were revealed with Godin reagent. A shift in the nature of the compounds that are synthesized and released to the extracellular medium by *M. rileyi* is observed among the extracts obtained in

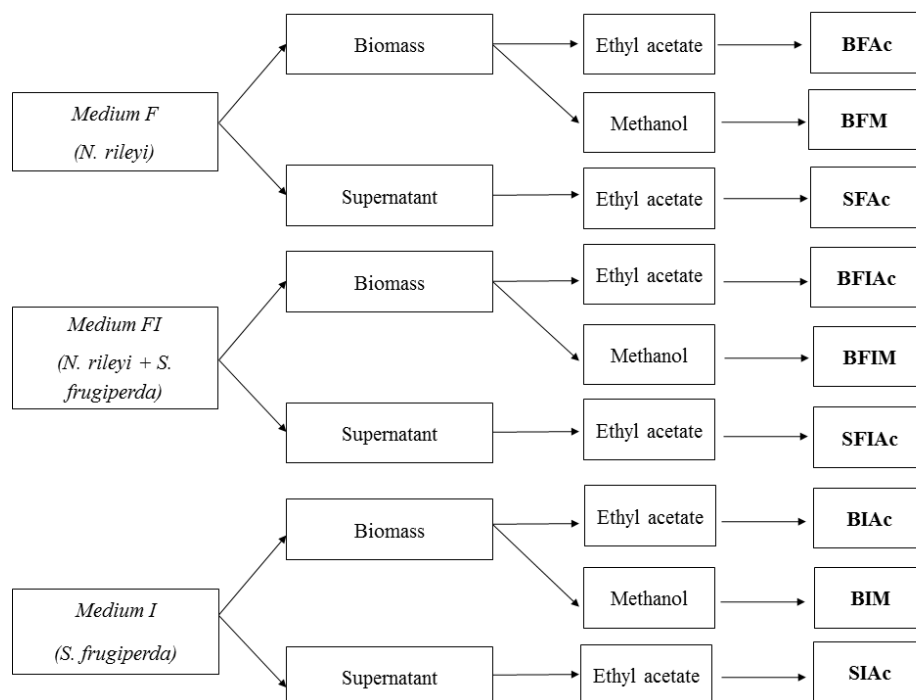


Figure 1: Culture of *Metarhizium rileyi* in presence and absence of insects and preparation of the extracts.

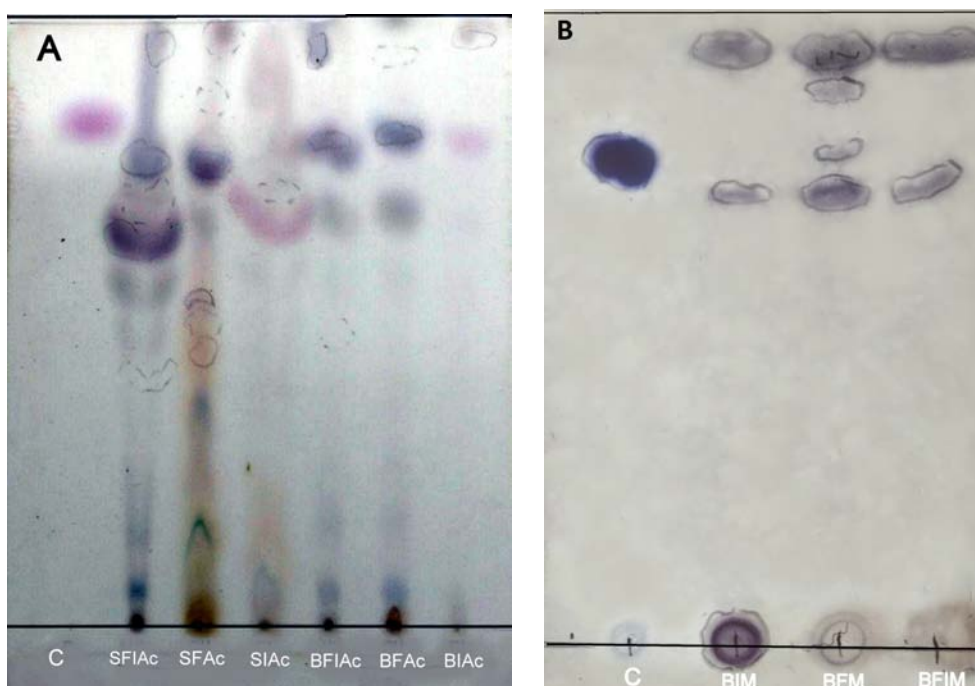


Figure 2: Phytochemical profile of *Metarhizium rileyi* (A) ethyl acetate extracts of biomass (BFIAc, BFAc, BIAc) and supernatant (SFIAc, SFAc, SIAc) and (B) methanolic extracts of the biomass (BFIM, BFM, BIM). TLC, Silica gel, chloroform:ethyl acetate, 7:3 v/v. Detection method: Godin reagent.

the absence and presence of *S. frugiperda*. SFAC profile reveals a pattern of brown spots correlated with flavonoids around the lower part of the plate (Retention factor $R_f < 0.5$), which is not visible in SFIAc (Figure 2A).

On the other hand, only SFIAc exhibited a violet spot on the upper part of the plate ($R_f 0.64$) (correlated with terpenoids). BFIAc profile reveals an intense violet spot with $R_f 0.93$, which is not visible in BFAc and BIAc (Figure 2A). These groups of compounds might be specifically synthesised in response to the presence of *S. frugiperda*, since the other eluates observed seems to be constitutively produced by the *M. rileyi* regardless of the insect presence. On the contrary, for the methanolic biomass extracts, a possible interruption of the synthesis of some compounds is observed by the fading out of two bands in BFIM compared to BFM (Figure 2B).

This is in agreement with studies on *Metarhizium*, a cosmopolitan Hypocrealean fungus used as a model of study for this order, where differential gene expression was observed depending on their living stage as saprophytes, epiphytes, pathogens, or even depending on their infection stage [26]. In addition, this result is correlated with the elicitation of secondary metabolites due to the insect addition observed in an entomopathogenic strain of *Aspergillus parasiticus* [27].

3.3. Antimicrobial Assay

3.3.1. Inhibition of the Bacterial Growth

Once the bacteria colonize a surface, it adheres reversibly and moves freely until a mature biofilm is formed. Since a minimum number of microorganisms are necessary for biofilm maturation, bacteria are required to the community from planktonic bacteria, or the population number is increased through replication of the existent bacteria [28]. As a result, inhibiting the bacterial growth would be an effective measure to prevent biofilm formation and the development of citrus cankers. Moreover, lowering the bacterial load on citrus would also reduce the probability of contagion on the field and during shipment.

In Table 2 is shown the effect of 100 $\mu\text{g/mL}$ of the biomass and supernatant extracts on the growth of three *Xanthomonas citri* subsp. *citri* (Xcc) strains. *M. rileyi* ethyl acetate biomass extracts with and without *S. frugiperda* fragments (BFIAc, BFAc) were able to significantly ($p < 0.05$) affect Xcc growth, with percentages of inhibition ranging from 37.2 to 66.1%. The insect control (BIAc) exhibited percentages of inhibition lower than 15%. This effect on the bacterial growth might be related to the presence of antimicrobial metabolites on the cuticular surface as part of the insect's natural defense mechanisms against pathogens [13].

Table 2: Inhibitory Capacity of 100 µg/mL *M. rileyi* Extracts of Three *Xanthomonas citri* subsp. *citri* Strains' Growth

Sample	Extract type	Sample	Xcc20 Inhibition (%)	Xcc29 Inhibition (%)	Xcc42 Inhibition (%)
Biomass	Ethyl acetate	BFIAC	37.2 ± 5.3 ^{a,b,c}	56.1 ± 7.1 ^{b,c,d}	52.1 ± 6.7 ^c
		BFAc	48.5 ± 4.1 ^{c,d}	66.1 ± 2.6 ^{c,d}	48.5 ± 5.9 ^{b,c}
		BIAc	15.0 ± 8.7 ^{a,b}	13.2 ± 9.9 ^{a,b}	10.2 ± 11.0 ^{a,b,c}
	Methanol	BFIM	18.1 ± 2.1 ^{a,b}	23.3 ± 6.4 ^a	14.6 ± 8.9 ^{a,b,c}
		BFM	32.1 ± 2.2 ^d	46.6 ± 6.7 ^{b,c}	3.7 ± 7.0 ^{a,b}
		BIM	12.0 ± 3.0 ^{a,b,c}	11.6 ± 4.8 ^{a,b,c}	2.3 ± 7.7 ^a
Supernatant	Ethyl acetate	SFIAC	1.7 ± 7.9 ^a	8.4 ± 6.2 ^a	7.2 ± 8.9 ^{a,b}
		SFAc	9.4 ± 3.7 ^a	5.8 ± 6.9 ^a	5.1 ± 8.9 ^{a,b}
		SIAC	14.6 ± 3.0	12.5 ± 5.8 ^{a,b,c}	13.2 ± 10.8 ^{a,b,c}
Antibiotic		Ciprofloxacin*	100.0 ± 2.2 ^e	100.0 ± 7.9 ^d	100.0 ± 1.0 ^d

Ethyl acetate extracts of biomass: Fungi+Insect (BFIAC), Fungi (BFAc), Insect (BIAc).

Methanolic extracts of biomass: Fungi+Insect (BFIM), Fungi (BFM) and Insect (BIM).

Ethyl acetate extracts of supernatant: Fungi+Insect (SFIAC), Fungi (SFAc) and Insect (BIAC).

*Ciprofloxacin 50 µg/mL.

Values (mean ± SD, n=3) in the same column followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

As for the biomass methanolic extracts, a significant effect was only observed among *M. rileyi* extracts in the absence of the insect (BFM), with inhibition percentages of 32.1 and 46.6% for Xcc20 and Xcc29 strains, respectively. BFM showed a noticeable growth inhibitory effect compared to BFIM for the Xcc29 strain (Table 2). While the supernatant extracts reduced Xcc growth less than 15%. On the other hand, the copper resistant strain Xcc42 was only inhibited by BFIAC and BFAc.

These results would also suggest that *M. rileyi* might constitutively synthesize biologically active compounds against the three Xcc strains with antimicrobial activity that remain attached to *fungus* surface.

3.3.2. Inhibition of Biofilm Formation

Biofilm is a bacterial community that develops attached to a surface embedded in an extracellular matrix [29]. It has been demonstrated that Xcc strains that have a defective capacity to form biofilm exhibit a reduced growth on their host and have a limited ability to induce canker symptoms on citrus leaves [2]. The effect of the biomass and supernatant extracts on the biofilm formation by three *Xanthomonas citri* subsp. *citri* strains (Xcc20, Xcc29, and Xcc42) was determined on LB medium. BFIAC and BFIM showed the highest level of inhibition ($38.2 \pm 7.2\%$ and $46.6 \pm 1.1\%$, respectively, at 100 µg/mL) on the biofilm formation by Xcc20 (Table 3). It is noticeable that while BFIAC had a mild inhibitory capacity at this concentration, BFAc and BIAc did not show significant inhibitory activity. BFM,

on the other hand, was not able to inhibit Xcc20's capacity to form a biofilm, even though BFIM was one of the strongest extracts. This would suggest a shift on *M. rileyi* secondary metabolism associated with its cellular wall in response to *S. frugiperda*.

A similar response, as described before, was observed for Xcc29's biofilm formation. As seen in Table 3, even though BFAc is not able to inhibit biofilm formation by Xcc29, BFIAC is one of the most active extracts along with BFIM ($31.5 \pm 3.7\%$ and $43.4 \pm 3.7\%$, respectively). These results would also suggest a change in *M. rileyi* secondary metabolism in response to its host, represented by a shift of the secondary metabolism toward the synthesis of molecules attached to *M. rileyi*'s surface. This is in accordance with the variations observed in the TLC profiles (Figure 2).

Supernatant extracts, SFIAC, SFAc, as well as the biomass extract BFIM, exhibited the highest inhibitory capacity of Xcc42 biofilm formation after 48 h of incubation, with a percentage of inhibition of $28.9 \pm 5.2\%$, $22.1 \pm 9.5\%$, and $27.4 \pm 5.0\%$, respectively (Table 3). In this case, the active metabolites appear to be both surface molecules and medium polarity metabolites that are excreted to the extracellular medium.

Moreover, it was observed that *S. frugiperda*'s extracts (BIAc, BIM, and SIAC) were not able to significantly affect the biofilm formation by all the Xcc strains evaluated. Therefore, the inhibitory effect of the

Table 3: Inhibitory Capacity of 100 µg/mL *M. rileyi* Extracts on Biofilm Formation (48h) by three *Xanthomonas citri* subsp. *citri* Strains

Sample	Extract type	Sample	Xcc20 Inhibition (%)	Xcc29 Inhibition (%)	Xcc42 Inhibition (%)
Biomass	Ethyl acetate	BFIAC	38.2 ± 7.2 ^c	31.5 ± 3.7 ^b	0.0 ± 4.2 ^a
		BFAc	9.1 ± 8.5 ^{a,b}	3.0 ± 2.9 ^a	4.0 ± 4.3 ^a
		BIAc	11.3 ± 5.5 ^{a,b,c}	3.3 ± 5.7 ^a	2.3 ± 5.1 ^a
	Methanol	BFIM	46.6 ± 1.1 ^b	43.4 ± 3.7 ^b	27.4 ± 5.0 ^a
		BFM	5.3 ± 5.9 ^a	15.8 ± 4.3 ^{a,b}	6.5 ± 8.6 ^a
		BIM	0.0 ± 3.3 ^a	9.3 ± 6.3 ^a	3.6 ± 7.9 ^a
Supernatant	Ethyl acetate	SFIAc	23.0 ± 5.0 ^a	18.8 ± 4.6 ^a	28.9 ± 5.2 ^a
		SFAc	27.1 ± 2.6 ^{a,b,c}	17.6 ± 9.5 ^{a,b}	22.1 ± 9.5 ^a
		SIAc	10.1 ± 2.6 ^{a,b,c}	9.2 ± 5.5 ^{a,b}	9.6 ± 11.4 ^a
Antibiotic		Ciprofloxacin*	100.0 ± 3.3 ^d	100.0 ± 3.7 ^c	100.0 ± 4.0 ^b

Ethyl acetate extracts of biomass: Fungi+Insect (BFIAC), Fungi (BFAc), Insect (BIAc)

Methanolic extracts of biomass: Fungi+Insect (BFIM), Fungi (BFM) and Insect (BIM)

Ethyl acetate extracts of supernatant: Fungi+Insect (SFIAc), Fungi (SFAc) and Insect (SIAc).

*Ciprofloxacin 50 µg/mL.

Values (mean ± SD, n=3) in the same column followed by a different letter are significantly different (Tukey test, $p \leq 0.05$).

extracts obtained from *M. rileyii* exposed to *S. frugiperda* might be related to compounds produced by the fungus.

Fungi synthesize a great array of metabolites that could effectively counteract its host defense mechanisms as it was identified for different entomopathogenic fungi [15]. The results are in agreement with Cartagena *et al.* (2014) [27] who reported an enhanced capacity to inhibit the bacterial biofilm formation by an entomopathogenic fungus extract when it is exposed to its host. Our results are promising as a potential source of natural biofilm regulator since studies carried out by Petrocelli *et al.* (2012) [30] on *Xanthomonas axonopodis* pv. *citri* mutants, which had the capacity to produce an altered biofilm, revealed that those mutants exhibited variations in the microscopical structure of the colonies, depleted motility, and reduced capacity to attach to leaves and develop cankers *in vivo*.

3.3.3. Inhibition of *Xanthomonas citri* subsp. *citri* Motility

Swimming allows Xcc to move across its host to colonize it through stomata and wounds, and to search for adequate sites for adhesion and biofilm formation. It is also important during the initial attachment to host, and biofilm maturation and dispersal [31]. Malamud *et al.* (2011, 2013) [28,32] demonstrated a reduced swimming capacity on Xcc and *X. axonopodis* pv. *citri* mutants, which have a defective capacity to form a biofilm. This result was also confirmed by Sena-Velez

et al. (2015) [31]. It was also observed that alterations in the flagellar structure result in reduced swimming, biofilm formation and reduction of virulence [28].

For the present work, we evaluated the capacity of extracts obtained from *M. rileyi* biomass and supernatant to inhibit Xcc strain's swimming capacity on a semi-solid LB medium.

The irregular nature of the motility patron exhibited by Xcc20 and Xcc 29, made it difficult to quantify the extracts' inhibitory capacity; nonetheless, as seen in Figure 3 all extracts were capable of inhibiting the motility of Xcc20. As for Xcc29, SFAc and BFIM had the strongest inhibitory effect on the motility, followed by BFIAC (Figure 4). It is noticeable that *M. rileyi* extracts were able to inhibit Xcc strains motility independently of the presence of *S. frugiperda*.

As seen in Figure 5, Xcc42, a copper resistant strain, shows a different swimming pattern compared to the other strains. SFIAc completely inhibited Xcc42 motility, whereas SFAc inhibited 89% of this strain swimming capacity. Both *M. rileyi* + *S. frugiperda* biomass extracts (BFIAC and BFIM) exhibited a low capacity to inhibit Xcc42 motility (8% and 12%, respectively). As for the extracts obtained from *M. rileyi* biomass, only BFAc was able to affect Xcc42 motility (32% of inhibition), while BFM not only did not influence the bacterial motility but also induced *M. rileyi*'s growth. This result would suggest that the active components that affect Xcc42 swimming capacity are constitutively

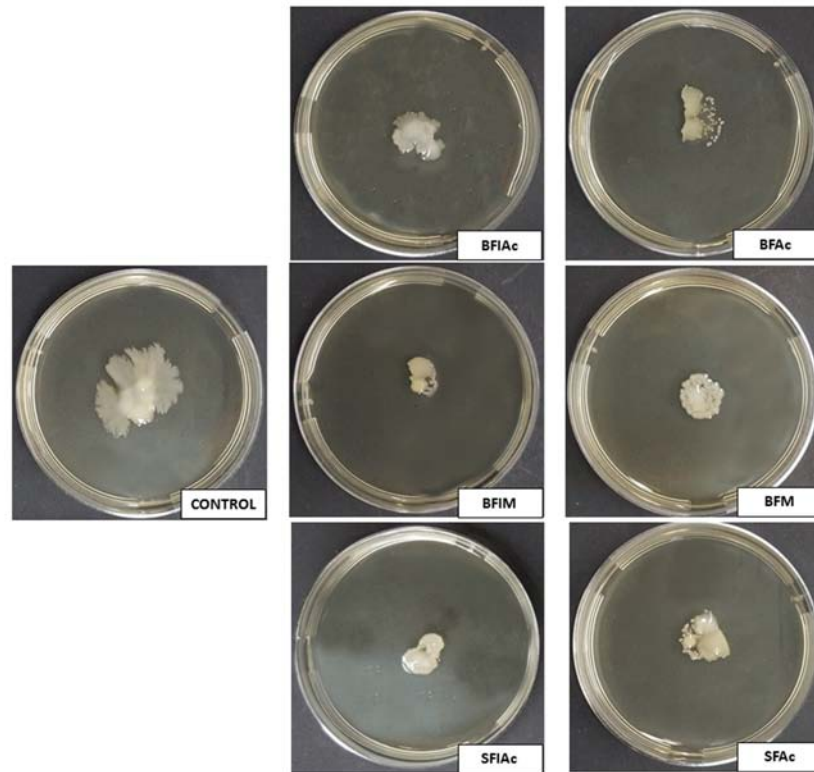


Figure 3: Effect of 100 $\mu\text{g}/\text{mL}$ of the Biomass ethyl acetate extracts: Fungi+Insect (BFIAc), Fungi (BFAC), Insect (BIAc), of the Biomass methanolic extracts: Fungi+Insect (BFIM), Fungi (BFM) and Insect (BIM), and of the Supernatant ethyl acetate extracts: Fungi+Insect (SFIAc), Fungi (SFAC) and Insect (BIAc) on *Xanthomonas citri* Xcc20 motility. Control: DMSO:Water (1:1).

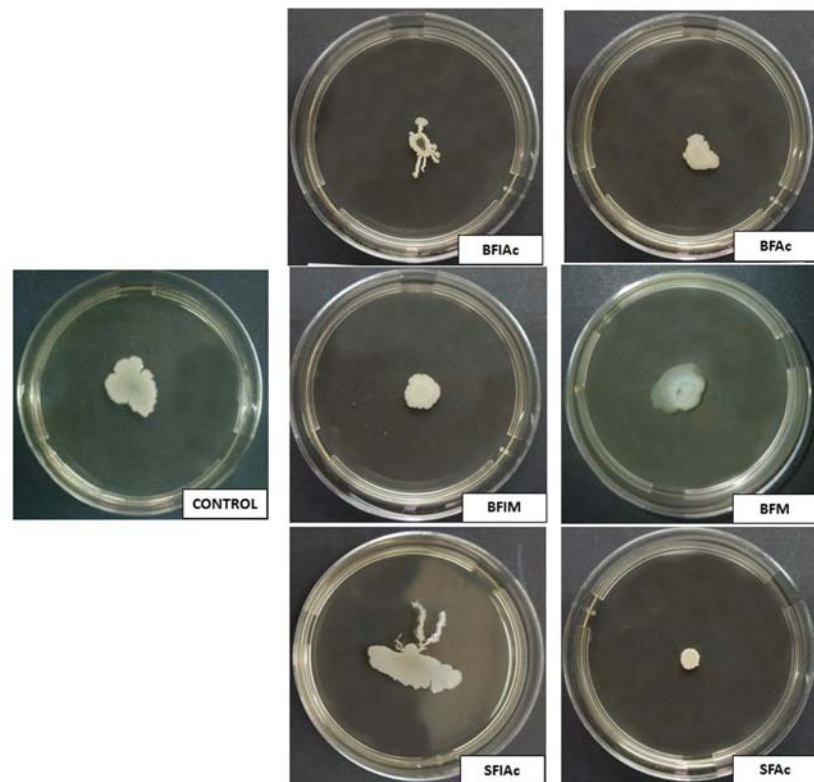


Figure 4: Effect of 100 $\mu\text{g}/\text{mL}$ of the Biomass ethyl acetate extracts: Fungi+Insect (BFIAc), Fungi (BFAC), Insect (BIAc), of the Biomass methanolic extracts: Fungi+Insect (BFIM), Fungi (BFM) and Insect (BIM), and of the Supernatant ethyl acetate extracts: Fungi+Insect (SFIAc), Fungi (SFAC) and Insect (BIAc) on *Xanthomonas citri* Xcc29 motility. Control: DMSO:Water (1:1).

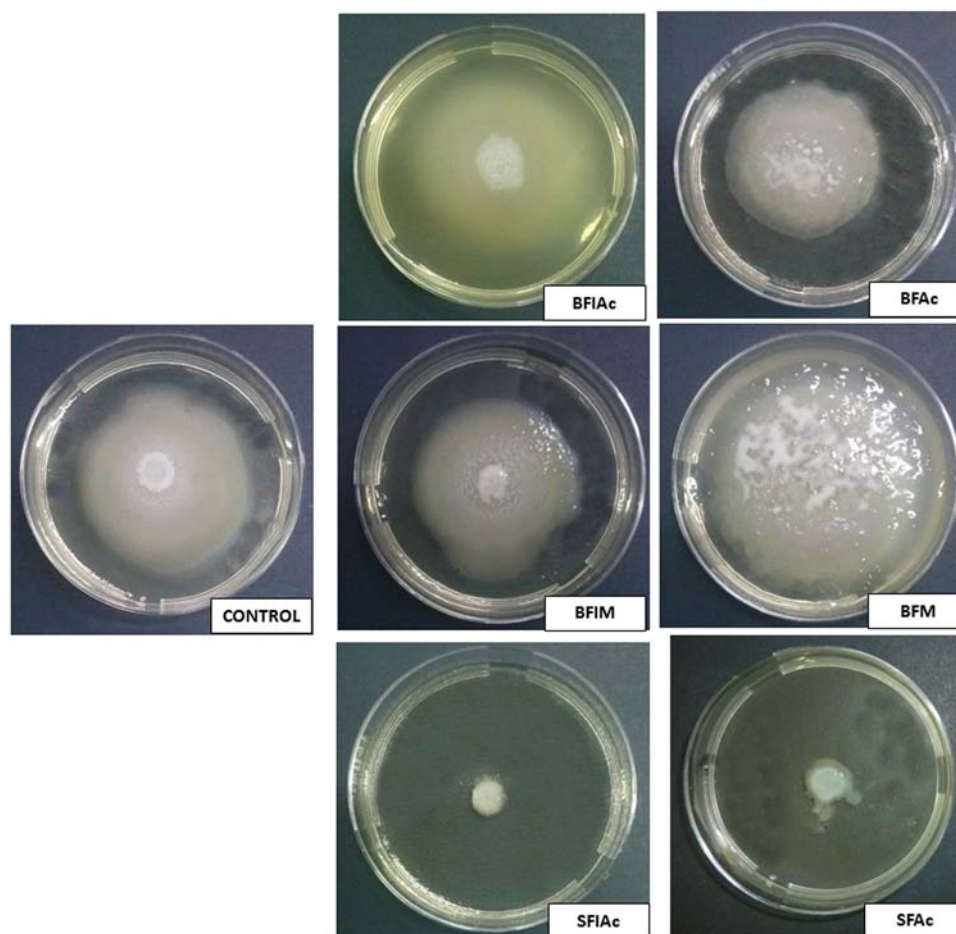


Figure 5: Effect of 100 µg/mL of the Biomass ethyl acetate extracts: Fungi+Insect (BFIac), Fungi (BFAc), of the Biomass methanolic extracts: Fungi+Insect (BFIM), Fungi (BFM), and of the Supernatant ethyl acetate extracts: Fungi+Insect (SFIac), and Fungi (SFAc) on *Xanthomonas citri* Xcc42 motility. Control: DMSO:Water (1:1).

synthesized by *M. rileyi* and excreted to the extracellular medium.

The effect exhibited by all the extracts on these three Xcc strains shows their potentiality to be used as preventive agents since alterations in Xcc's motility have an important impact on its capacity to form and mature biofilm. *Xanthomonas* presents a distinctive biofilm structure, characterized by a mushroom-like structure formed by clusters of cells, which are separated by water channels necessary for nutrients flow throughout the biofilm structure [11]. Alterations in this structure were observed in *Xanthomonas axonopodis* pv. *citri* mutants lacking on important components of the flagellum, which resulted in thinner biofilms and reduced virulence [28].

3.4. Inhibition of Cankers Development

Xcc's virulence factors act in coordination and are required at different times of the infective cycle; therefore, it is necessary to evaluate the effect of the

extracts in Xcc's natural host to determine their effectiveness under natural conditions where all virulence factors would be expressed.

For this study, we selected the most sensitive strain, *Xanthomonas citri* subsp. *citri*'s Xcc20, to carry out *in vivo* assays on Duncan grapefruit leaves. As seen in Figure 6, leaves treated with supernatant extracts SFIac, SFAc, as well those that were coated with biomass extracts BFIM, and BFM do not develop any pathologic symptom after infection with the aforementioned Xcc strain. *In vitro*, all these extracts have in common a strong effect against Xcc20 swimming capacity, which could be regarded as a possible mechanism of action for these extracts, since it has been proved that there is strong correlation between motility, biofilm formation, and *Xanthomonas*' virulence [28]. In concordance with our results, Malamud *et al.* (2011, 2013) [28,32] and Felipe *et al.* (2018) [33] have demonstrated the crucial role of flagella on biofilm formation and cankers development by *Xanthomonas citri* pv. *citri* and *Xanthomonas*



Figure 6: Effect of Biomass ethyl acetate extracts: Fungi+Insect (BFIac), Fungi (BFAC), of the Biomass methanolic extracts: Fungi+Insect (BFIM), Fungi (BFM), and of the Supernatant ethyl acetate extracts: Fungi+Insect (SFIAc), and Fungi (SFAc) on *Xanthomonas citri* Xcc20 infected grapefruit leaves.

vesicatoria, which would explain the inhibitory activity observed in our extracts for the *in vivo* assays. In another experiment, Li and Wang (2014) [2] noted that foliar application of two biofilm inhibiting compounds (D-leucine and 3-indolylacetonitrile) reduced this bacteria population in plant and cankers occurrence.

Xcc virulence depends upon the expression of various virulence factors that dictate numerous events occurring before and after infection. Biofilm formation, growth, motility, and attachment to surfaces, are among the most important virulence factors in the infective cycle of *Xanthomonas* species. The extracts that affect bacterial growth, but mainly, biofilm formation and swimming are correlated as cankers protector substances. Biofilm has major importance as a protective mechanism against environmental stress

and plants' defensive system (like H_2O_2 and phenolic compounds) [34]. It has been suggested that biofilm not only plays an important role in the epiphyte survival of Xcc, but also in the early stages of infection and attachment of the bacteria to xylem vessels [20,2].

Even though more assays would be needed to further demonstrate the effectiveness of *M. rileyi*'s compounds to control Xcc's cankers development on citrus leaves, these results are promising to develop an alternative and natural preventive strategy to control this citrus illness of great economic worth.

4. CONCLUSION

M. rileyi biomass ethyl acetate extracts (BFIac and BFAC) had a strong effect on Xcc strains growth. On the other hand, the presence of *S. frugiperda* as an

elicitor significantly increased *M. rileyi*'s inhibitory capacity to form biofilm as seen by the stronger effect exhibited by BFIAc and BFIM compared to BFAc and BFM, respectively. A different behavior was observed for the copper resistant strain, Xcc42, as for its biofilm formation and swimming capacity since the most active samples were the supernatant extracts (SFIAc and SFAC). Further assays would be needed to explain these results. Moreover, our results also suggest that the most important factor for *in vivo* canker development is the inhibition of Xcc's motility since all the extracts that were able to inhibit canker development *in vivo* were able to inhibit Xcc20's motility *in vitro*. The fact that the extracts can prevent cankers development *in vivo* indicates they are promising sources of metabolites to prevent the development of this citrus disease.

CONFLICT OF INTEREST

The authors have declared that there is no conflict of interest.

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