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In vitro antagonism of *Clonostachys* sp. against disease associated fungi in economically important crops

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ABSTRACT

Objectives/Background. The objective of this work was to evaluate the *in vitro* antagonistic capacity of a strain of *Clonostachys* sp. against five species of fungi associated with diseases in economically important crops.

Materials and Methods. Five fungal species associated with crop diseases were tested: *Alternaria alternata, Colletotrichum kahawae, C. musae, Fusarium oxysporum* and *F. solani*. Dual cultures were performed with five replicates plus controls. Growth was recorded every 24 hours, until 360 hours were completed. Interactions were determined, the degree of antagonism and the percentage of colonization was calculated. Statistical analyses were performed with a generalized linear model (GLM).

Results. All species evaluated showed antagonism of the overgrowth type. The degree of antagonism was classified into three classes, with class two being present in three of the species. The percentage of colonization was 100% at 216 h for three of the species and 264 h for the other two. There was no significant difference in the percentage of colonization ($p = 0.0073$), but there was a significant difference in the time of invasion ($p < 0.0001$).

Conclusion. Dual assays to test the antagonistic effect *in vitro* form the basis for the selection of candidates for biological control of fungi.

Key words: antagonism, biological control, mycoparasites.

Introduction

The genus *Clonostachys* comprises fungi with diverse lifestyles, including destructive mycoparasites that are used as biocontrol agents against plant pathogen fungi, as well as other species with other types of ecological associations (Schroer, 2001); few studies related to this genus have been published in Mexico, although *Clonostachys rosea* was recently cited to be parasiting avocado in Puebla (Cóyotl-Pérez *et al*., 2022), along with *C. chloroleuca,* causing wilting in chickpea (*Cicer arietinum*) in Sinaloa and Baja California (Cota-Barreras *et al*., 2022). Out of the mycoparasitic species, the most widely studied and used as a biological control agent is *Clonostachys rosea* (Funck and Dubey, 2022), which acts against several plant pathogens such as the genera *Alternaria*, *Botrytis*, *Bipolaris*, *Drechslera*, *Moniliophthora*, *Phytophthora*, *Rhizoctonia*, *Rhynchosporium* and *Sclerotinia* (Sun *et al*., 2020). One of the alternatives for the control of diseases caused by plant pathogens is the use of biocontrols, which are sustained in the ability of certain fungal groups to inhibit the growth of others, using hydrolitic enzymes such as chitnases and glucanases, giving them a distinctive advantage by letting them aim directly at the hyphae of other fungi, degrading cell walls more effectively (Moore *et al*., 2020). Due to this, investigations on this group of fungi are important to discover new possibilities of biological control.

In *Clonostachys*, 11 mycoparasitic species have been reported (Schroers, 2001). To date there are no reports of *Clonostachys* as a mycoparasite in Veracruz, therefore the aim of this work was to evaluate the degree of antagonism *in vitro* of an isolated *Clonostachys* sp. strain against five strains of phytopathogenic fungi: *Alternaria alternata*, *Colletotrichum musae*, *C. kahawae*, *Fusarium oxysporum* and *F. solani.*

Clonostachys was isolated (Senanayake *et al*. 2020) as a mycoparasite of an ascomycete (*Lachnum petridophyllum*) living in the mesophilic forest of Veracruz. For the isolation, PDA medium (MCD LAB) was used, incubated for five days at 26 °C. The strain is located in the strain collection of the Centro de Micología Aplicada Research (CIMA) of the Universidad Veracruzana under the code CIMA-F-168. The strain was viewed under the Primo Star Iled compound microscope (Carl Zeiss, Oberkochen, Germany) and primary and secondary conidiophores were measured (Schroers *et al*., 2001). Since the strain has morphological characteristics that do not correspond to any of the cited mycoparasitic species of *Clonostachys*, it is currently undergoing molecular identification.

Accrding to Comporota (1985), the first phase to select a strain with the potential for biocontrol is to evaluate its ability to invade other fungi. To corroborate this, five species of fungi related to diseases in previously studied crops were selected: *Alternaria alternata* (CIMA- F-056; Trigos *et al*., 2008); *Colletotrichum kahawae* (CIMA- F-078; isolated by A. Salinas from the Bola de Oro estate, in Coatepec,

Veracruz); *Colletotrichum musae* (CIMA- F-012; donated by COLPOS); *Fusarium oxysporum* (CIMA-F-068; Adame *et al*., 2015); *F. solani* (CIMA-F-127; Lagunes *et al*., 2015), all of which were deposited in the s CIMA-UV strain collection. The strains were planted in a PDA medium and incubated in a cultivation oven (BG-E-71) at 26 °C for seven days.

The antagonistic activity was evaluated using dual cultures, placing two discs, 4 mm in diameter, in 90 mm Petri dishes with PDA medium. A PDA disc with *Clonostachys* sp. mycelial growth was placed on one side, and on the other, the fungus related to the cultures. The distance of separation between the discs was 50 mm and 15 mm from the edge of the dish. Five repetitions were carried out for each confrontation assay.

As a control, each one of the evaluated fungi were sown without the presence of *Clonostachys* sp., as well as the *Clonostachys* sp. strain without the presence of other fungi. Both the controls and the dual cultures were incubated at 26 ºC. Measurements of the growth (in millimeters) of both fungi in confrontation and the control were taken every 24 hours until 360 hours were completed (15 days).

The interaction between the fungi *in vitro* was observed and described, with particular attention to the production of reproductive structures, pigmentation and mycelium morphology. The degree of antagonism (Table 1) was determined using the scale by Bell *et al*. (1980).

Using the growth measurements, the percentage of colonization was determined, following the formula by Camporota (1985): C= DT/DE/100, where DT is the distance covered by *Clonostachys* sp. on the axis that separates the points of planting and DE is the distance between both (5 cm). The colonization was considered effective when the percentage was greater than 50% (Rollan *et al*., 1999). The statistical analyses were carried out using the R software (R Core Team). The normality of the data was corroborated using the Shapiro-Wilks. Subsequently, a generalized linear model (GLM) analysis was carried out.

The strain under study displayed a mycoparasitic-like antagonistic behavior against the five fungal species evaluated, with *Fusarium solani* corresponding to

Table 1. Qualitative scale of the degree of antagonism of *Clonostachys* sp. and the evaluated fungi associated to diseases.*

Degrees	Antagonistic capacity
1	*Clonostachys* exceeded the fungus and covered the entire surface of the medium.
2	*Clonostachys* exceeded at least 2/3 of the surface of the medium.
3	*Clonostachys* and the fungus have each colonized approximately half of the surface of the medium
4	The fungus colonized at least 2/3 of the surface of the medium
5	The fungus completely exceeded *Clonostachys*.

*Modified from Bell *et al*. (1980).

class 1; *Alternaria alternata, Colletotrichum musae* and *C. kahawae*, to class 2; *Fusarium oxysporum*, to class 3, following the scale proposed in Table 1. In none of the cases was an inhibition halo observed, so the interaction observed corresponds to the type of "overgrowth," according to the classification by Bertrand *et al.* (2013).

The percentage of colonization of *Clonostachys* sp. was 100% after 216 h against *A. alternata*, *C. kahawae* and *F. solani*, whereas for the species *C. musae* and *F. oxysporum*, it was after 264 hours under *in vitro* conditions (Table 2). Although the colonization was effective, no significant differences were found in the percentage of colonization among the phytopathogens evaluated (p= 0.0073). Nevertheless, the time of invasion did have a significant effect on the percentage of colonization for each fungus, as indicated by the value of $p < 0.0001$ obtained in the GLM analysis.

Table 2. Percentage of growth of *Clonostachys* sp. against the fungi.

Time (hours)	**Average *A. alternata* versus *Clonostachys* sp.**	**Average of the percentage of growth of *Clonostachys* sp.**
24	7.2-6.4	12.7
72	25.6-21.2	42.0
120	40.7-35.6	69.5
168	45.3-45.0	87.8
216	46.5-53.8	100
264	49.4-60.8	100
312	51.8-70.6	100
360	53.2-78.0	100
Time (hours)	**Average *C. kahawae* versus *Clonostachys* sp.**	**Average of the percentage of growth of *Clonostachys* sp.**
24	4.8-7.4	14.7
72	19.9-21.6	43.2
120	40.2-36.6	73.2
168	45.8-49.6	99.2
216	50-58.8	100
264	50-65.6	100
312	50-70.8	100
360	50-76.0	100
Time (hours)	**Average *C. musae* versus *Clonostachys* sp.**	**Average of the percentage of growth of *Clonostachys* sp.**
24	6.4-7.1	14.2
72	41.8-20.7	41.4
120	46.8-35.5	71.0
168	46.8-42.3	84.7
216	46.8-48.7	97.3
264	46.8-53.1	100
312	46.8-56.7	100
360	46.8-65.8	100

Table 2. Continue

Time (hours)	Average *F. solani* versus *Clonostachys* sp.	Average of the percentage of growth of *Clonostachys* sp.
24	4.5-7.4	14.7
72	15.2-21.0	42.0
120	23.2-36.6	73.2
168	30.7-45.0	90.0
216	33.3-52.8	100
264	34.0-59.4	100
312	34.9-69.2	100
360	34.9-75.0	100
Time (hours)	**Average *F. oxysporum* versus *Clonostachys* sp.**	**Average of the percentage of growth of *Clonostachys* sp.**
24	9.5-7.9	15.9
72	32.9-21.1	42.3
120	45-32.9	65.8
168	50-40.8	81.5
216	50-46.8	93.6
264	50-54.8	100
312	50-57.8	100
360	50-58.8	100

On the other hand, the evaluated fungi display different growth rates and particular characteristics for instance, in the case of *Alternaria alternata* (Figure 1), after 168 hours, it produced a bright yellow pigment in the culture medium, which intensified into an orange color. According to Scott and Stoltz (1980), the diffuse yellow pigments in the medium are related to the production of toxins such as altertoxin II in *A. alternata.*

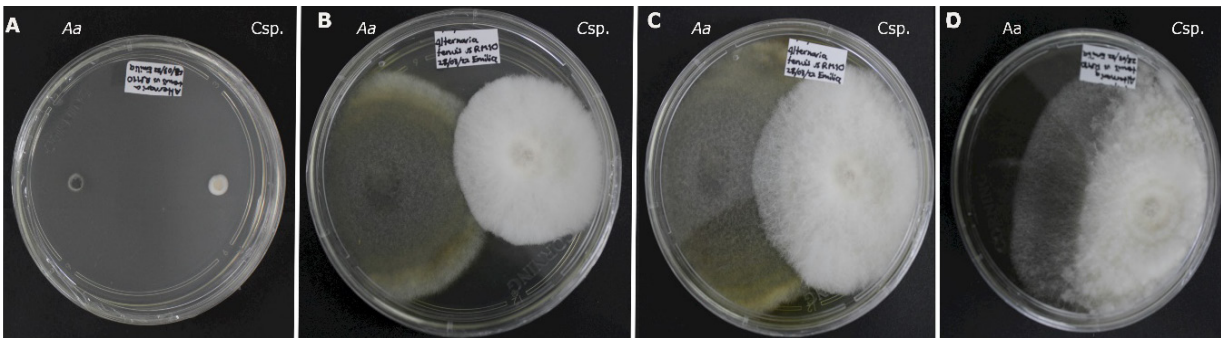

Figure 1. *In vitro* antagonism of *Clonostachys* sp. (Csp.) against *Alternaria alternata* (Aa). A: After 24 hours of evaluation. B. 120 hours of evaluation. C. 216 hours of evaluation. D. 360 hours of evaluation.

After144 hours in *C. kahawae*, a brown pigment was observed in the center of the colony, which intensified as the antagonist grew on the fungus (Figure 2). Likewise, during the interaction between *Clonostachys* sp. agains *C. musae*, the growth of the antagonist was observed on the acervuli produced by *C. musae* (Figure 2). This process is highly important, since it can contribute significantly to the disruption of the cycle of the pathogen, since acervuli form at the end of the infection cycle, breaking the cuticle of the plant to emerge and continue with asexual reproduction (da Silva *et al.*, 2020).

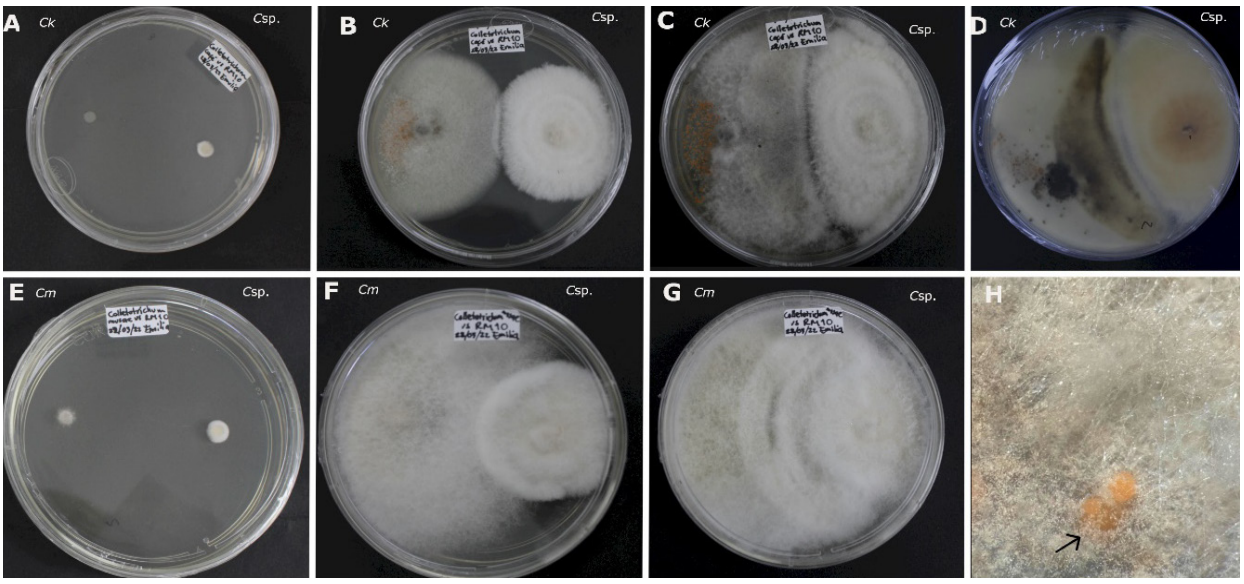

Figure 2. *In vitro* antagonism of *Clonostachys* sp. (Csp.) against *Colletotrichum kahawae* (Ck) and *Colletotrichum musae* (Cm). A-D) *Colletotrichum kahawae* (Ck). A: 24 hours after evaluation. B: 192 hours of evaluation. C: 360 hours of evaluation. D: reverse Petri dish at 360 hours of evaluation. E-H) *Colletotrichum musae* (Cm) against *Clonostachys* sp. (Csp.). E: 24 hours of evaluation. F: 192 hours of evaluation. G: 360 hours of evaluation. H: Conidiophores of *Clonostachys* sp. growing on acervuli of *C. musae*.

Although the *Clonostachys* genus has been the object of study in relation to its antagonistic activity, there are few investigations centered on its interaction with the species of the *Colletotrichum* genus. Peters *et al.* (2020) isolated *Clonostachys rosea* as the endophyte of the açai plant (*Euterpe oleracea*) and proved its ability to inhibit the growth of *Colletotrichum gloeosporioides,* the causal agent of anthracnose. This study presents, for the first time, the antagonistc evaluation against two species of the *Colletotrichum* genus that had not been previously evaluated.

In the case of the interaction between *F. solani* and *Clonostachys* sp. after 168 hours, the aerial mycelium of *Clonostachys* sp. changed its morphology, forming a

convex colony with a fimbriated edge, unlike the control and trials with other fungi. Masses of conidia with an aqueous consistency were observed on the edge after 240 horas (Figure 3).

Some of the characteristics between *F. oxysporum* and *Clonostachys* sp. was that, after 168 hours, they both colonized half of the Petri dish, with their edges touching. During this period, the *F. oxysporum* pigments intensified, changing from a lilac color at the beginning of the assay to a dark purple color in the middle with bright edges at the end of the experiment, keeping the same color as the control (Figure 3).

Several studies conducted with the same genera tested in this trial showed that the *in vitro* and *in vivo* antagonistic activity of 10 strains of *Clonostachys* and *F. circinatum* in *Pinus radiata* seedlings (Moraga *et al.,* 2011) was variable. The strains inhibited *F. circinatum* by up to 23% in *in vitro*, but not *in vivo*, in which the percentage of survival of the seedlings infected by the fungus increased. It has also been proven that the endochitinase enzymes participate in the degragation process of cell walls of species such as *Fusarium culmorum* (Mamarabadi *et al*., 2008).

The interactions observed betweem *Clonostachys* sp. and the fungi under study displayed distinctive charateristics, such as the production of masses of conidia and a different mycelial morphology during the trial, particularly in *F. solani*. In addition,

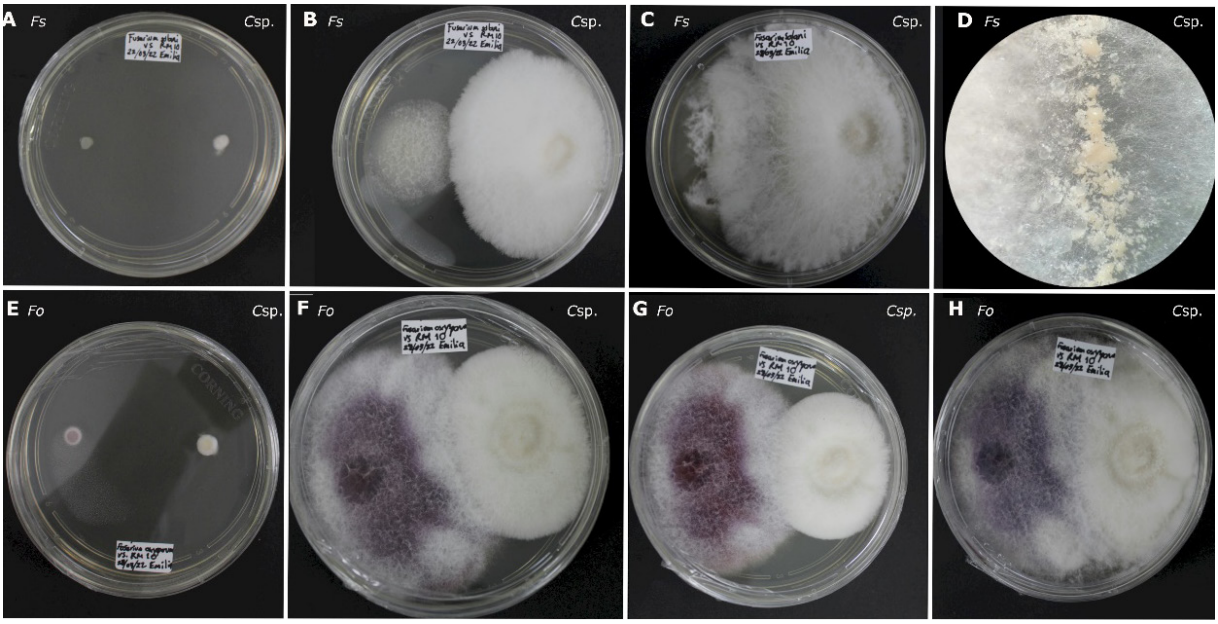

Figure 3. *In vitro* antagonism of *Clonostachys* sp. against *Fusarium* spp. A-D) *Fusarium solani* (Fs) against *Clonostachys* sp. (Csp.) A: after 24 hours of evaluation. B: at 96 hours of evaluation. C: at 360 hours of evaluation. D: masses of aqueous conidiophores at the margin of *Clonostachys* sp. E-H) *Fusarium oxysporum* (Fo) against *Clonostachys* sp. (Csp.) E: 24 hours of evaluation. F: 144 hours of evaluation. G: 192 hours of evaluation. H: 360 hours of evaluation.

a variation was recorded in the speed of invasión of each fungus evaluated. These differences can be attributed to factors such as transcriptomic regulation and the ability of *Clonostachys* to discriminate between the species it will mycoparasitize, as seen in the case of *C. rosea* (Nygren *et al*., 2018).

Dual trials to verify the antagonistic effect of species at an *in vitro* level constitute the basis to seek better candidates for the biological control of fungi that cause diseases in economically important crops. Given the marked noticeable activity of the *Clonostachys* sp. strain against all the fungi evaluated, it emerges as a promising candidate for future research to evaluate its *in vivo* effectiveness.

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