Control of *Fusarium* spp. and *Bacillus subtilis* through metabolites of *Xenorhabdus bovienii* mutualist of *Steinernema feltiae*

Control de *Fusarium* spp. y *Bacillus subtilis* mediante metabolitos de *Xenorhabdus bovienii* mutualista de *Steinernema feltiae*

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Abstract

The antimicrobial activity of metabolites of *Xenorhabdus bovienii* (Enterobacteriaceae) was evaluated, a mutualist of the entomoparasite nematode *Steinernema feltiae* (Filipjev, 1934 Cephalobina, Steinernematidae) over *Fusarium* spp. and *B. subtilis*. *X. bovienii* was purified from *Galleria mellonella* larvae infected with *S. feltiae* and extracted in a bioreactor. Its growth was sampled at intervals of 4 h for 96 h of culture, a time on which metabolites were extracted by using six methods: organic compounds, proteins, indole compounds, methanol, column chromatography and butanol. The metabolites extracted from *X. bovienii* at four concentrations were faced with *B. subtilis* and *Fusarium* spp. in tomato seeds, Santa Clara variety. The inhibition halo (mm) was assessed for *B. subtillis* and for *Fusarium* spp. in the presence of macro, microconidia, and chlamydospores. Phytotoxicity was assessed *X. bovienii* showed higher growth and the presence of metabolites in the first 16-20 h of incubation up to 40 h. The maximum inhibition occurred at concentrations of 100% and early sampling. In *Fusarium* spp. Mycelial growth inhibition coincided with a lower presence of macro, microconidia, and chlamydospores. No phytotoxic effects of the metabolites tested were observed. The potential of *X. bovienii* was tested for control of *Fusarium* spp. and *B. subtilis* as a microorganism sensitive to changes by these metabolites.

Key words: *Bacillus subtillis,* biological control, *Fusarium* spp., secondary metabolites, *Xenorhabdus bovienii*.

Resumen

En el estudio se evaluó la actividad antimicrobiana de metabolitos de *Xenorhabdus bovienii* (Enterobacteriaceae), mutualista del nematodo entomoparásito *Steinernema feltiae* (Filipjev, 1934) clamidosporas, Cephalobina, Steinernematidae sobre *Fusarium* spp. y *Basillus subtilis*. *Xenorhabdus bovienii,* se extrajo a partir de larvas de *Galleria mellonella* infectadas con *S. feltiae* e incubadas en biorreactor. Su crecimiento fue medido en intervalos de 4 h durante 96 h de cultivo, en las cuales se extrajeron metabolitos por las metodologías: compuestos orgánicos, proteínas, indol, metanol, columna para cromatografía y butanol. Se utilizaron cuatro concentracciones de metabolitos de *X. bovienii* que se probaron contra *B. subtillis* y *Fusarium* spp., en semillas de tomate variedad Santa Clara mediante la medición del halo de inhibición (mm) por la presencia de macro y microconidias y clamidosporas. *Xenorhabdus bovienii* presentó mayor crecimiento y presencia de metabolitos entre 16 y 40 h de incubación. La máxima inhibición ocurrió con concentraciones de 100% durante los primeros muestreos. En *Fusarium* spp., la inhibición del crecimiento micelial coincidió con menor presencia de macro- y microconidias y clamidosporas. No se encontraron efectos fitotóxicos de los metabolitos probados sobre las plántulas de tomate.

Palabras clave: *Bacillus subtillis*, control biológico, *Fusarium* spp., metabolitos secundarios, *Xenorhabdus bovienii*.

Introduction

Tomato (*Solanum lycopersicum* L.) is a vegetable of economic importance in Valle del Cauca, Colombia, where it is grown in monoculture by small farmers, a system that has affected the increment of pests and diseases which significantly increase production costs. Several *Fusarium* species cause considerable damage to the stem and root of the tomato plant, a fungus whose resistance structures (chlamydospores) can remain in the soil for years. To control pathogens in tomato is frequent the uncontrolled application of high doses of synthetic chemical products, however these mutate and adapt easily to new conditions, which affect the environment (Infoagro, 2011). Therefore, it is urgent the use of biological control practices, that compared with the use of synthetic chemical products, are less polluting for the environment, since these controllers and antagonists have several strategies for action; including the production of secondary metabolites present in organisms or their symbionts, which can be isolated and multiplied for use in this type of control (Xu, 1998).

The *S. feltiae – X. bovienii* complex has a wide host range and skills to search and introduce its mutualistic bacteria within the body of the insect, causing septicemia within the following 24-48 h and surpassing the immune reactions presented in the hemolymph as cellular defense (Parada *et al.*, 2006; Martinez, 2010).

The strain Colombia lifecycle of the complex *S. feltiae - X. bovienii* has been widely documented by Triviño (2006) and Martinez (2010). The capacity of biological control of this complex is based on the fact that the bacteria produce secondary metabolites with antibiotic capacity, which act as alternative of defense and communication with other species. In *X. bovienii* growth phases FI and FII are distinguished, which in culture medium differ in their morphology, physiology and expression (Xu, 1998; Triviño, 2006).

In pure cultures or solutions of *Xenorhabdus* species, sources of natural antibiotics have been found such as indoles, xenocoumacins xenorhabdins and which are of use for both the medical field and in agriculture (Xu, 1998). Although interest and demand has grown for the use of entomoparasitic nematodes for the control of these pests in Colombia, in the last decade (Parada *et al.*, 2006), little has been explored on the antimicrobial activity of the bacteria associated with *Xenorhabdus* spp. and *Photorhabdus* spp., symbionts of *Steinernema* spp. and *Heterorhabditis* spp., respectively. This work aims to contribute to the development of this alternative of biological control and evaluate the antimicrobial activity of some secondary metabolites from *X. bovienii*, the mutualistic bacteria of the entomoparasitic nematode *S. feltiae* (Filipjev, 1934) on isolates of *Fusarium* spp., a resident of soil and rhizosphere of tomato crop.

Materials and methods

The work involved three stages: (1) obtaining pure cultures of the microorganisms *Fusarium* spp., *X. bovienii* and *B. subtilis*, the latter being the control bacteria given its high susceptibility to metabolites from *X. bovienii* (Xu, 1998); (2) pathogenicity tests to select highly pathogenic isolates of *Fusarium* spp. on tomato variety Santa Clara. In this same step growth kinetics was determined and bacterial secondary metabolites from the broth of *X. bovienii* were obtained by six extraction methodologies; and (3) the antimicrobial activity of the metabolites on *B. subtilis* and *Fusarium* spp. was evaluated, its toxicity on seeds and tomato seedlings of variety Santa Clara.

The field samplings were conducted in 2008 in the municipalities of Santa Elena (SE), Guacarí (Gu), Media Canoa (MC), El Bolo (B), and Toro, in the department of Valle del Cauca, and Puerto Tejada (PT) in the department of Cauca. Soil samples, taken at the crop site and consisting of the

rhizosphere and plant material with possible symptoms of Fusarium were analyzed in the Laboratory of Microbiology at the Universidad Nacional de Colombia, Palmira. In the isolates *Fusarium* spp. was identified, which in pure form was used for the pathogenicity tests in tomato seeds from variety Santa Clara (Duarte, 2007). As an indicator a pure culture of *B. subtilis* was used, facilitated by the Universidad Nacional de Colombia, Bogotá, and highly sensitive to metabolites of *X. bovienii* (Xu, 1998).

The obtaining of the secondary metabolites from *X. bovienii* was accomplished starting from infected *Galleria mellonella* larvae with *S. feltiae* and incubated in a 3 l bioreactor with NBTA medium; subsequently growth kinetics was estimated at sampling intervals every 4 h for 96 h of culture. From the material collected metabolites were extracted with the methods proposed by Xu (1998): 1. Organic Compounds, 2. Proteins, 3. Indole compounds, 4. Methanol, 5. Chromatography column and 6. Butanol.

X. bovienii metabolites were tested against *B. subtilis* and *Fusarium* spp., at four concentrations (100, 75, 50 and 25%) for each extraction methodology, wherein sensidiscs were used (filter paper, Whatman) for 40 seconds during which the formation of inhibition zones was observed (mm), which were measured 24 h after planting and incubated with both organisms. Furthermore, the presence of macro and microconidia and chlamydospores present in the culture media CLA (Carnation Leafpiece- Agar) and SNA (Spezielller Nahorstoffarer Agar) was observed (Lara *et al.*, 2010).

To study the phytotoxicity of these metabolites on pathogens, tomato seeds impregnated with each solution were divided into four groups which were placed in Petri dishes containing each of the four dilutions, depending on the time of each treatment. After this period the seeds were sown in sterile soil and taken to a controlled growth chamber. Monitoring was conducted for ten days till obtaining 10 cm length seedlings aproximately, taking into account the formation of discolored areas and the presence of spots. Treatments were arranged in a complete randomized design, data were analyzed with SAS® 9.2 program and comparisons among means by Duncan test ($P < 0.05$).

Results and discussion

Isolation of the microorganisms

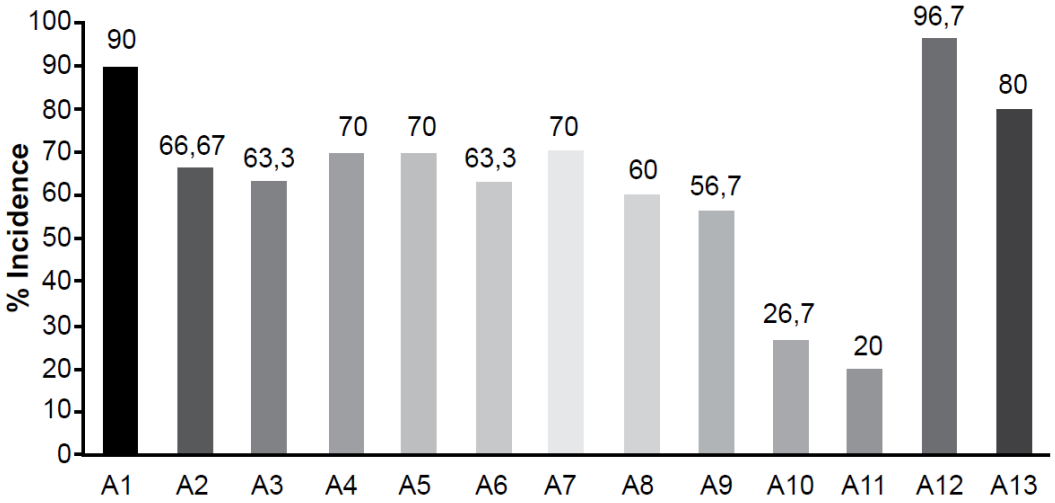
According to the damage scale proposed by Duarte (2007) - 0 = healthy seed; 1 = damage to a portion of the seed in contact with the mycelium; 2 = seed coat invaded by mycelium and sclerotia, but the seedling appears to be healthy; 3 = integument of the seed free of the fungus, but the seedling is infected; 4 = integument of the seed and seedling infected; 5 = infected and not germinated seed - in this study, from thirteen isolates, six had severe incidence of pathogens.

The isolates of *Fusarium* spp., obtained from samples taken in the department of Valle del Cauca showed the highest occurrence being more incident A12 (SETM1M4), A1 (GUM1R2), A13 (SETMF1M6), A4 (GUM5R2), A5 (GUM6R1) and A7 (BTa1). The degree of incidence on these last three was similar ($P> 0.05$). Meanwhile B. subtilis strain was identified as C-4 (Figure 1).

Growth kinetics and metabolite extraction from *X. bovienii*

The pure culture of *X. bovienii* obtained in the bioreactor showed a differential growth during the 96 hours of evaluation. In the growth curve, the steeper slope occurred between 16 and 20 h followed by a growth phase with a constant slope until 32 h; from this point and up to 72 h growth attenuation was followed until stabilization up to 96 h. After drying in a rotary evaporator at the end of 36 h, 1 ml of an oily consistency liquid and amber color was obtained, which increased to a maximum average of 1.5 ml, to finally achieve a maximum concentrate of 500 µl.

Figure 1. Percentages of incidence of the thirteen isolates of *Fusarium* spp. on tomato seeds, variety Santa Clara.



A1: GuM1R2, A2: GuM3r, A3: GuM4, A4: GuM5R2, A5: GuM6R1, A6: MCM1, A7: BTa1, A8: BTU2, A9: BTa3, A10: PTM5R3, A11: PTM5R2, A12: SETM1M4, A13: SETMF1M6 (Figure 1), where reference is made to the sampling municipalities such as: GU = Guacari, MC = Media Canoa, B = Bolo, PT = Puerto Tejada, and SE = Santa Elena.

Antimicrobial activity of the secondary metabolites on *Bacillus subtillis*.

The metabolite extraction method, the tested concentrations, and the time of exposure, as the interaction of the latter showed differences ($P < 0.05$) (Table 1).

With the exception of proteins (Vela, 2009), metabolites extracted by the five methodologies significantly decreased the growth of this bacteria (Table 2). The greatest inhibition occurred when pure metabolite was used without dilution (100%) and the highest antibiosis on *B. subtilis* appeared when the metabolites were extracted from *X. bovienii* between 8 and 12h.

About *Fusarium* spp.

The extraction method and the concentration of metabolites of *X. bovienii* as the exposure time exhibited differences ($P < 0.05$) in the halos of inhibition (Table 3). As with *B. subtilis*, the halos of inhibition of the isolates showed greater effect when applied 100% concentration

Table 1. Analysis of variance on the inhibitory effect of *Bacillus subtilis* on secondary metabolites of *X. bovienii* obtained by different extraction methods, times and concentrations.

Source	D.f.	*Bacillus subtillis*	
		MS	Pr > F
Extraction method	4	1.349	<.0001
Concentration (C)	3	2.064	<.0001
Time (T)	17	0.129	<.0001
C x T	51	0.023	0.0099
Mean	—	1.136	—
VC (%)	—	9.4	—

Table 2. Averages (mm) of the inhibition halo of growth of *Bacillus subtilis* B in response to the exposure of secondary metabolites obtained by different extraction methods analyzed.

Method	Average
Butanol	1.381 a*
Column[a]	1.281 a
Indole	1.261 a
Organics	1.269 a
Methanol	0.574 b

* Averages followed by the same letters are not statistically different (P> 0.05), 5% probability.
a. Chromatography column.

and extractions from 0 to 40 h.

Structure formation and sporulation of isolates of *Fusarium* spp.

Significant differences (P < 0.05) among isolates and culture media (CLA and SNA) were found for the expression of macro and microconidia, but not for chlamydospores, in which only changes from culture media were detected. The incubation time during which the secondary metabolites were extracted significantly affected these three structures. For chlamydospores, the more favorable culture media was CLA, as it lacks carbohydrate sources, which stimulates the formation of structures of resistance during the first 20 h. For macro and microconidia, interactions did not show differences (P > 0.05) indicating that the presence and expression of these structures were only dependent on culture medium and extraction time metabolite (Tables 4 and 5).

Metabolite phytotoxicity

The metabolites applied in concentrations between 25% and 100% did not affect crop development of tomato variety Santa Elena. According to Xu (1998) in the life cycle of *X. bovienii* in symbiosis with the nematode *S. feltiae*, once the bacteria is released by the nematode, its population multiplies rapidly and produces several metabolites that exceed the insect immune system that serves as host, causing death by septicemia within the following 24-48 h. The inhibitory response to metabolites extracted from *X. bovienii* in all tested on *B. subtilis* methodologies and isolates of *Fusarium* spp. (excluding proteins) was particularly high in the early hours of incubation (4, 8, 12, 16, 20 h).

Table 3. Average halos of inhibition (mm) on the isolates of *Fusarium* spp. vs. metabolites of *Xenorhabdus bovienii* extracted by five methods

Isolate	Metabolite extraction method				
	Butanol	Methanol	Organics	Indole	Column[a]
BTU2	0.80 a*	0.73 b	0.59 c	0.54 d	0.48 e
GMU1R2	0.52 d	0.73 b	0.80 a	0.56 cd	0.58 c
GM5R2	0.94 a	0.64 c	0.73 c	0.81 b	0.95 a
GM6R1	0.51 d	0.74 b	0.88 a	0.58 c	0.56 c
SETM1M4	0.49 c	0.79 b	1.13 a	0.49 c	0.43 d
SETMF1M6	0.45 e	0.73 b	0.81 a	0.62 c	0.53 d

*Averages followed by the same letters are not statistically different (P > 0.05) level probability of 5%. a. Chromatography column.

Table 4. Analysis of variance of the effect of two culture media tested in the expression of macro-, microconidia and chlamydospores of six isolates of *Fusarium* spp.

Source	D.f.	Macroconidia		Microconidia		Chlamydosp.	
		MS	Pr > F	MS	Pr >F	MS	Pr > F
Isolate (I)	5	440933.8	<0.0001	1943895.3	0.1622	1312	0.1622
Medium (M)	1	43447.0	<0.0001	153365.7	0.0023	7743.2	0.0023
I x M	8	4893.0	0.282	27347.6	0.001	3528.2	0.001
Time (T)	17	7856.6	<0.0001	39055.5	0.0003	2260.2	0.0003
T x I	85	264.2	0.0245	13574.5	< 0.0001	1650.4	< 0.0001
T x M	17	1690.8	0.6040	6146.6	0.0729	1284.5	0.0729
T x I x M	85	2127.3	0.2684	5794.4	< 0.0001	1543.5	< 0.0001
Mean	324	—	645	—	172.8	—	—
VC(%)	13.6	—	14.0	—	16.6	—	—

Table 5. Effect of Time (h) of extraction of secondary metabolites on the expression of macro-, microconidia and chlamydospores of six isolates of *Fusarium* spp.

Time (hours)	Av.	Time (hours)	Av.	Time (hours)	Av.
96	352.3 a*	96	706.2 a	32	189.2 a
72	339.2 ab	72	678.6 ab	4	188.8 a
80	336.0 ab	80	672.2 abc	88	179.6 ab
94	336.0 ab	94	671.9 abc	96	178.6 abc
56	335.7 ab	56	671.4 abc	80	176.1 abc
32	334.8 ab	32	669.5 abc	48	175.9 abc
88	334.2 ab	88	668.6 abc	72	175.6 abc
48	330.1 abc	48	660.3 abcd	44	173.3 bc
44	328.3 bcd	44	656.6 bcd	56	172.4 bc
4	326.1 bcd	40	646.2 bcd	64	172.3 bc
40	323.1 bcd	36	634.9 bcde	40	171.6 bc
12	309.6 cde	12	619.1 def	28	170.7 bc
28	308.6 cde	28	617.2 def	24	165.5 bc
20	308.2 cde	20	616.4 def	8	165.5 bc
24	308.1 cde	24	616.2 def	12	164.2 bc
8	305.8 de	16	597.1 ef	16	162.7 c
16	298.5 e	4	581.3 f	20	162.3 c

*Averages followed by the same letters are not statistically different (P > 0.05) level probability of 5%. a. Chromatography column.

Xenorhabdus bovienii quickly digests the insect host and makes it available for use as food for the nematode *S. feltiae*; it also produces secondary metabolites that prevent infection and proliferation of opportunistic organisms (fungi, bacteria and even other nematodes) in the body of the parasitized larva. However, the presence of these antimicrobial compounds depends on the hours of sampling and extraction methods, identifying some metabolites as xenorhabdines, nematophines and xenocoumacines, among others (Xu, 1998).

The results in this study show the biocontrol potential for *Fusarium* spp., by *X. bovienii* considering possibly, not only isolated metabolites but the culture medium as a whole (medium compounds rich in nutrients, metabolites secreted by the bacterium and even the population of *X. bovienii*).

Conclusions

- With a 95% probability it can be assured that B. subtilis is highly sensitive to the antibiotic activity of *X. bovienii* and was valid to use these bacteria as an indicator organism.
- The effectiveness of the obtained secondary metabolites on the fungus *Fusarium* spp. was observed, affecting its resistance structures.
- However, this fungus is still presented as a constraint on various crops including tomato. It is required, therefore, to develop new alternatives for biological control with other organisms that can replace or complement the chemical synthesis controllers, as proposed in this paper.
- The secondary metabolites of the bacteria *X. bovienii* showed no phytotoxic activity on seeds and seedlings of tomato variety Santa Clara.
- Regardless of the method of extraction, the maximum antibiosis on *B. subtilis* appeared when the metabolites of *X. bovienii* were extracted between 8 and 12 h, at which phase I of bacteria growth occur and the highest quantity of secondary metabolites with antibiotic properties are expressed.

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