Mycopath (2016) 14(1&2): 15-19

In vitro antagonism of *Bacillus* strains against *Fusarium* species

Nazario Francisco Francisco, *Gabriel Gallegos Morales, Yisa Maria Ochoa Fuentes, Juan Carlos Delgado Ortiz and Francisco Daniel Hernandez Castillo

Department of Parasitology, Universidad Autónoma Agraria Antonio Narro, Saltillo, Coahuila, México.
**Corresponding author's email: ggalmor@uaaan.mx*

Abstract

Antagonist strains of *Bacillus subtilis*, *B. amyloliquefaciens*, *B. liquefaciens* and *B. pumilus* were evaluated for their ability to control *Fusarium verticilliodes*, *F. oxysporum*, *F. acuminatum*, *F. solani*, *F. proliferatum* and *F. oxysporum* f. sp. *melonis* isolated from garlic (*Allium sativum* L.), melon (*Cucumis melo* L.), and pepper plants (*Capsicum annuum* L.). To evaluate the antagonistic effect of different *Bacillus* species, mycelia plugs from the edges of actively growing fungal cultures were placed in the center of Petri dish containing potato dextrose agar. *Bacillus* species were inoculated at an equidistant cardinal point. After 168 h, strong growth inhibition was observed with *B. pumilus* (38% with *F. verticilliodes*). However *F. oxysporum* isolated from pepper plant were less inhibited (7-9%) for the four *Bacillus* species. *Fusarium* growth inhibition by *B. pumilus* was evident after 120 h in dual culture.
Keywords: *Bacillus pumilus,* Biological control, Root disease.

Introduction

Fusarium spp. are responsible for many crop production losses around the world (Savary *et al.,* 2006; Summerell *et al.,* 2010). Various *Fusarium* species multiply to survive on individual seeds of crops without causing any visible symptoms (Ochoa-Fuentes *et al.,* 2012). To counteract the damage caused was chosen to treat seeds before planting (Tagne *et al.,* 2013). However, the presence of the inoculum in the fields represents a latent threat difficult to control with traditional alternatives as the application of chemicals (Funnell-Harris and Pedersen, 2011).

Among alternatives being studied, use of *Bacillus* strain has shown significant potential (Pérez-García *et al.,* 2011). It is generally recognized that *Bacillus* species show antagonistic potential against fungal phytopathogens by antibiosis, competition or exploitation. Successful control of *Fusarium* species has been achieved by various *Bacillus subtilis* isolates (Cao *et al.,* 2011). Some *B. subtilis* isolates were found less effective against *Fusarium* species in comparison with others *Bacillus* species due to mode of action exerted or the type of antifungal metabolite produced. Therefore, many studies have been conducted to find the best *Bacillus* strain or by inducing secondary metabolites production (Saini, 2012; Ola *et al.,* 2013). Foreign strain or commercial inoculants use has been shown less effective in other countries due to different edaphic or climatic conditions. Therefore, isolation and screening of native strain is suggested (Calvo *et al.,* 2010). The aims of this work were to determine the ability of four *Bacillus* species to inhibit seven *Fusarium* species and to evaluate the ability of the best strain bacterium *in vitro*.

Material and Methods

Four species of *Bacillus* namely *B. subtilis*, *B. amyloliquefaciens*, *B. liquefaciens*, and *B. pumilus* were used obtained from the microbial collection of the Department of Plant Pathology at the Universidad Autónoma Agraria Antonio Narro. The identification of these strains was confirmed (Guillén-Cruz *et al.,* 2006). *Fusarium* species (*F. verticilliodes*, *F. oxysporum*, *F. acuminatum*, *F. solani*, *F. proliferatum* and *F. oxysporum* f. sp. *melonis*) were isolated from pepper plants and identified from available litaerture (Ochoa-Fuentes *et al.,* 2012).

In vitro antagonism tests

To evaluate the antagonistic effect of different *Bacillus* species, mycelia plugs (6 days growth) from the edges of actively growing fungal cultures were placed in the center of Petri dishes containing potato dextrose agar (PDA). The *Bacillus* species (48 h growth) were inoculated at

an equidistant cardinal point. Plates were sealed with tape and incubated at room temperature (27 ± 2 °C). Growth inhibition of the mycelium was recorded at 120 h after confrontation using digital Vernier. In addition, growth inhibition of the mycelium by the best *Bacillus* sp., was recorded from the fifth to the tenth day after confrontation.

The percentage growth inhibition of the test fungus was calculated using the formula: (R1-R2/R1) X 100; where R1 (control) represents the radial growth of the fungus sets without bacteria, and R2 represents the radial growth of the fungus in sets inoculated with the *Bacillus* species.

The experiments were conducted in triplicate. The means and standard deviations of the inhibition of radial growth were calculated. All data were analyzed by one way analysis of variance (ANOVA). Significant difference ($P \leq 0.05$) among the means was determined by Tukey´s test, using the software SAS software (SAS Institute, Gary, NC, USA).

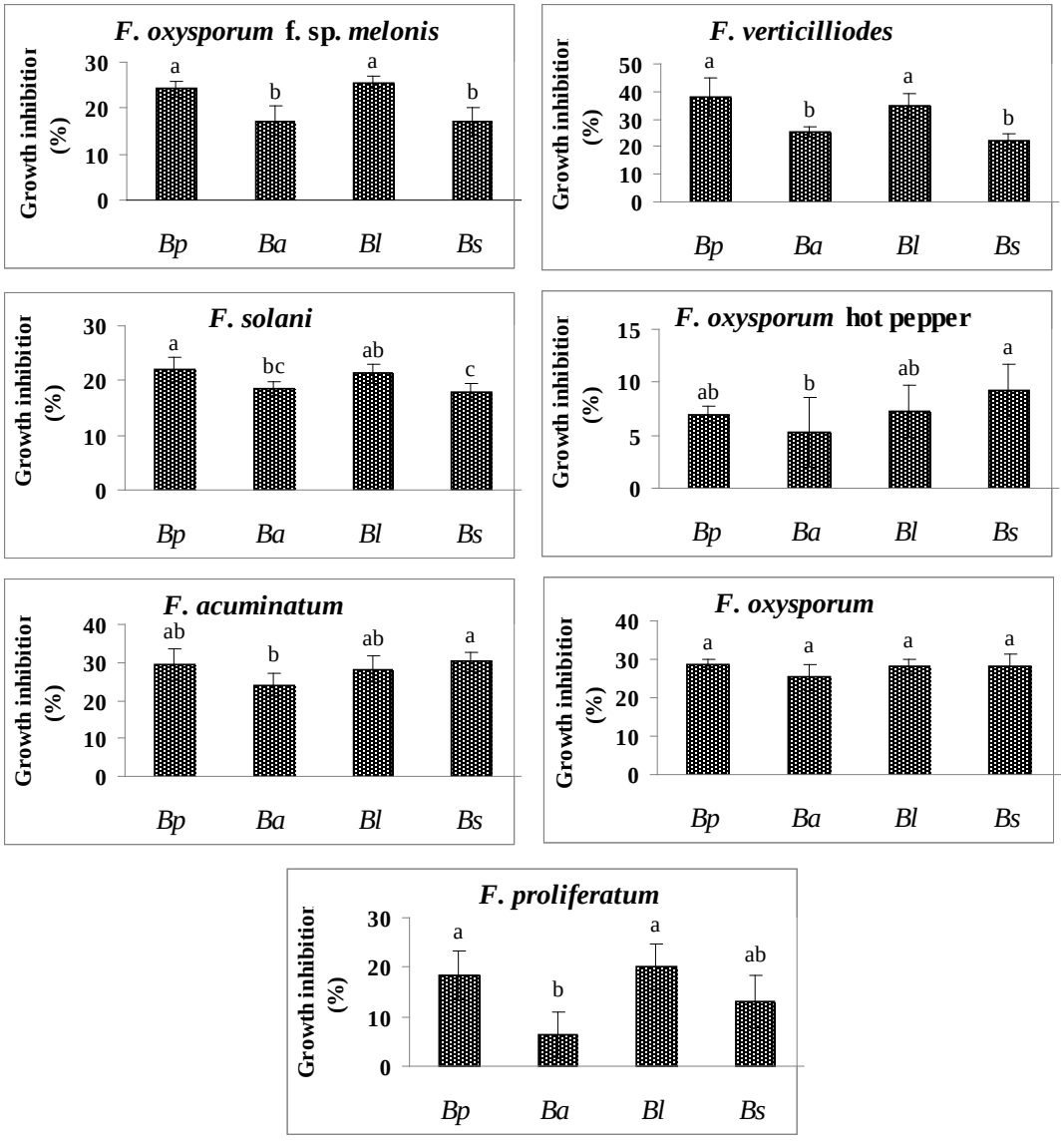

Fig. 1. Radial growth inhibition percentage of *F. verticilliodes*, *F. oxysporum*, *F. acuminatum*, *F. solani* , *F. oxysporum* f. sp. *melonis*, *F. oxysporum*, and *F. proliferatum* by *B. pumillus* (*Bp*), *B. amyloliquefaciens* (*Ba*), *B. liquefaciens* (*Bl*) and *B. subtilis* (*Bs*) after 168 h in dual culture.

Results and Discussion

Growth inhibition of *Fusarium* species by *Bacillus* spp. after 168 h confrontation is represented in Fig. 1. Among bacterial antagonist, *B. pumilus* and *B. liquefaciens* had significantly higher inhibitory effects; however the first one was the most efficient. *Bacillus* strains showed inhibition percentage of 5-38% in radial growth of fungi. Among different fungi all bacterial strains showed better antifungal potential (RGI: 20-40%) against *F. verticilliodes*. Pictorial observation revelead thick mycelial mat and plenty sporulation *F. acuminatum* in culture plate (Fig. 2).

Table 1 presents values of radial growth inhibition percentage by *B. pumilus* against *Fusarium* spp. Antifungal activity of bacterial strain of fungal growth was evident after 120 h confrontation. The values were different ranged from 0.4 ± 1.4% (*F. solani*) to 21.1 ± 4.0% (*F. acuminatum*). At 240 h the values ranged from 34.2 ± 1.2% (corresponding to *Foch*) to 43.0 ± 2.9% (corresponding to *F. acuminatum*). The values inhibition of fungal growth continued similarly with longer confrontation time. All tested *Fusarium* species showed the same inhibition patterns as the first assay with all *Bacillus* spp. (Fig. 2).

Table 1: Radial growth inhibition percentage (RGI) by *Baciilus pumilus* against *Fusarium* spp. after 120 to 240 h in dual culture.

	Radial growth inhibition (%)					
	120 h	144 h	168 h	192 h	216 h	240 h
Fv	11.8 ± 3.6†b	18.6 ± 3.5 bc	26.5 ± 3.8 b	35.5 ± 3.4 ab	39.6 ± 2.6 ab	38.5 ± 1.7 a-d
Fom	5.7 ± 3.2 bc	21.1 ± 3.6 b	29.4 ± 3.4 b	32.1 ± 3.2 b	37.0 ± 2.8 b	36.6 ± 2.2 cd
Fo	10.3 ± 2.6 b	21.3 ± 1.8 b	25.6 ± 1.6 b	35.2 ± 1.6 b	41.7 ± 1.2 ab	43.8 ± 1.4 a
Fs	0.8 ± 0.8 c	15.0 ± 0.6 bc	24.4 ± 0.2 b	30.3 ± 0.4 b	38.4 ± 0.6 ab	37.7 ± 0.9 b-d
Fp	2.0 ± 0.6 c	14.5 ± 0.3 c	25.8 ± 3.2 b	30.4 ± 2.9 b	38.2 ± 2.3 ab	40.9 ± 2.9 a-c
Fa	21.1 ± 4.0 a	33.3 ± 2.9 a	42.9 ± 2.7 a	42.2 ± 3.0 a	44.1 ± 3.5 a	43.0 ± 2.9 ab
Foch	0.7 ± 0.2 c	5.5 ± 0.0 d	13.3 ± 1.6 c	21.9 ± 0.6 c	29.3 ± 0.5 c	34.2 ± 1.2 d

†Standard deviation.
F. verticilliodes (*Fv*), *F. oxysporum* (*Fo*), *F. acuminatum* (*Fa*), *F. solani* (*Fs*), *F. oxysporum* f. sp. *melonis* (*Fom*), *F. oxysporum* isolated from pepper plant (*Foch*), *F. proliferatum* (*Fp*).

This work reveals that *B. pumilus* and *B. liquefaciens* can be used effectively against many *Fusarium* species; the first one most efficient than other. *In vitro* and *in vivo* evaluation of *B. subtilis* as successful antagonist towards various phytopathogenic fungi has been determined (Machado *et al.,* 2010; Todorova and Kozhuharova 2010; Cao *et al.,* 2011). In this study, the ability of *Bacillus* species to control *Fusarium* species may suggest the need for extent of inhibition by mixing *Bacillus* species or their volatile compound. For example, has been shown that dual application of *B. pumilus* and the *Glomus mosseae* improved the growth inhibition of fungal pathogens (Chakraborty *et al.,* 2011). On the other hand, it was observed effectiveness of *Bacillus* species was linked to their stage of growth. Sadfi and coworkers (2001) shown that *B. cereus* was most effective against *Fusarium* dry rot when applied as young cultures (24 h), however *B. thuringiensis* strains was most effective when applied as older cultures (48-72 h). Nevertheless, different studies revealed that *B. pumilus* produced different antifungal compound as "iturin" which inhibits growth of *Aspergillus* sp. and their aflatoxin producing (Cho *et al.,* 2009; Huang *et al.,* 2013). Growth inhibition *Fusarium* species by *B. pumilus* and *B. liquefaciens* probably was due by producing antimicrobial compound and are not affected by their stage growth.

Previous research work has shown *in vivo* assay that some of the strains of *Bacillus* sp. decreased incidence and severity of root rot caused by *Fusarium* sp. and *Rhizoctonia* sp. in pepper plant (Guillen *et al.,* 2006). We found that some *Bacillus* strains *in vitro* assay are effective against more than one *Fusarium* species. *B. pumilus* is more feasible for the antagonism of *Fusarium* species and could provide an alternative disease control for horticultural crops.

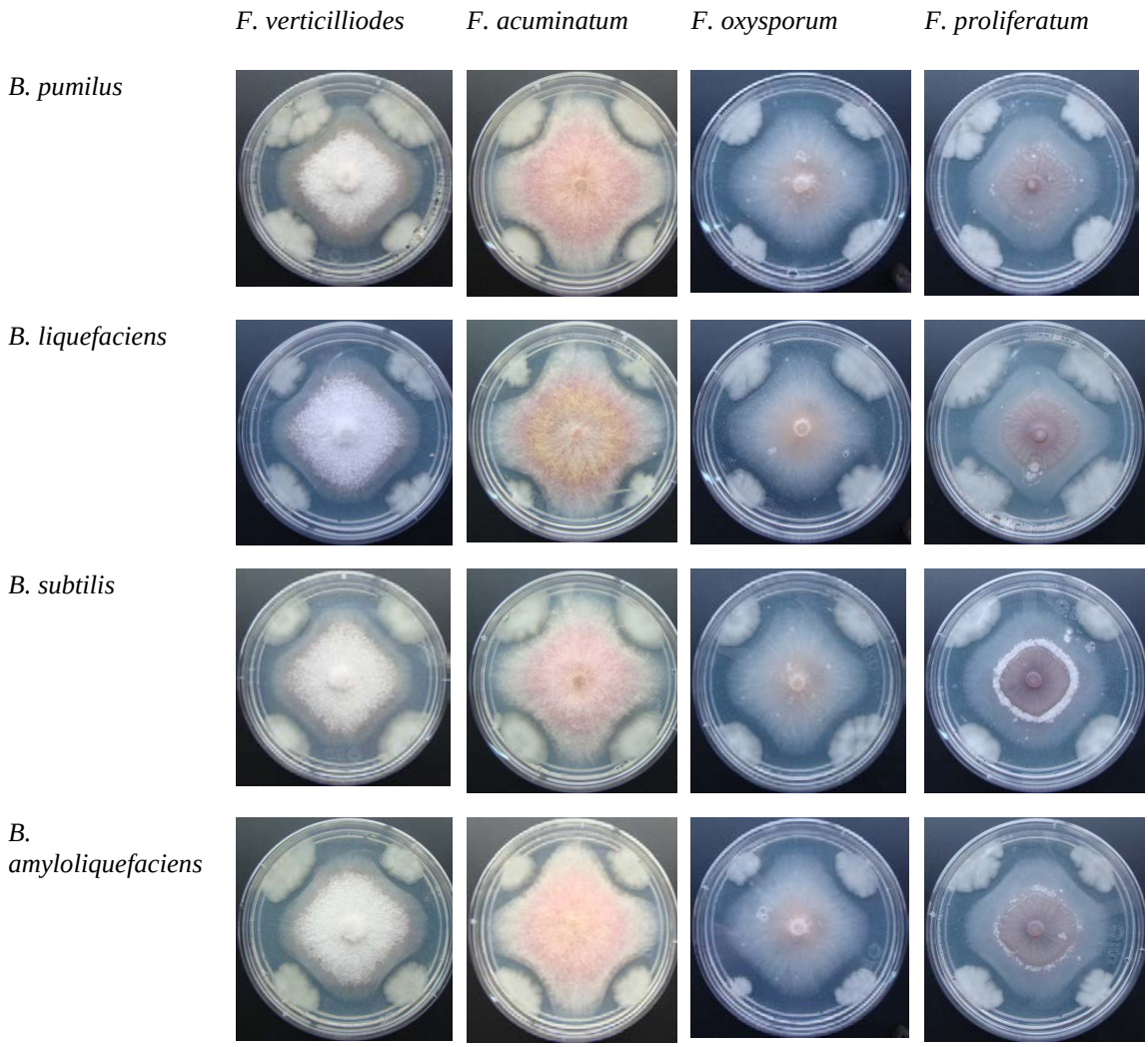

Fig. 2. Dual culture of *F. verticilliodes* (*Fv*), *F. acuminatum* (*Fa*), *F. oxysporum* (*Fo*), and *F. proliferatum* (*Fp*) with *B. subtilis*, *B. amyloliquefaciens*, *B. liquefaciens* and *B. pumilus* after 120 h.

Conclusion

All the *Bacillus* strains used *in vitro* experiments inhibited the *Fusarium* spp. However, the degree of antagonism of the strains for a given *Fusarium* sp. was variable and the inhibition degree depended on the *Bacillus* spp. None *Bacillus* strain used was effective against all *Fusarium* species, therefore, the screening of *Bacillus* strain in order to control of *Fusarium* species can be a way to optimize the better control.

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