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Antifungal Activity *in vitro* of Native *Bacillus* sp. Strains Against *Macrophomina phaseolina* (Tassi) Goid

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Abstract: The isolation of *Bacillus* strains with antifungal activity is important to carry out tests to protect economically important crops. We determined the *in vitro* activity of 13 native strains of *Bacillus* sp. isolated from soil against *Macrophomina pasheolina* using a dual assay in nutrient agar. All the strains showed inhibition of radial growth of *M. phaseolina* from 31-80%, with strains LUM B01 and B04 in a 80.8 and 75.9%, respectively with the highest antifungal activity. The crude extract of the strain of chitinases LUMB0 04 inhibited the growth of *M. phaseolina* by 30%. The strains of chitinolytic *Bacillus* sp. isolated from soils showed antifungal activity and have the potential to be used in biological control of *M. phaseolina*.

Key words: Antifungal activity, *Bacillus* sp. *Macrophomina phaseolina*, growth inhibitory, chitinase

INTRODUCTION

Macrophomina phaseolina is the causal agent of stem rot in various crops such as beans and soya bean. Phytopathogenic fungi are agents that cause economic losses in agriculture and to control these diseases is the use of common chemical pesticides, however has seen the development of fungicide resistance by fungal pathogens (Ishii, 2004), so antagonistic microorganisms such as bacteria and fungi are an alternative for controlling these pathogens.

Antagonism acts on antibiosis, competition or parasitism. Parasitism involves the production of hydrolytic enzymes as chitinases and β-1-3 glucanase (Woo *et al*., 2002; Compant *et al*., 2005; Prapagdee *et al*., 2008). The chitinases have an effect on the germination of conidia (Huang *et al*., 2005; Huang and Chen, 2008) and degrade the cell wall of pathogenic fungi (Gupta *et al*., 1995). Production of chitinolytic enzymes by fermentation can be performed using ground shrimp wastes (Reyes-Ramirez *et al*., 2004). Bacteria of the genus *Bacillus* has showed antifungal activity against phytopathogenic microorganisms (Cho *et al*., 2003; Feio *et al*., 2004; Huang *et al*., 2005; Hu *et al*., 2008). Isolation of strains of the genera *Bacillus* can be made directly from the soil and the activity of chitinases production can be determined by testing hydrolysis in medium with chitin (Rodas-Junco *et al*., 2009). These bacteria have the advantage of being already adapted to the environment where they can be applied as biological control. Studies of *in vitro* antifungal activity of the dual rate should be measured in a culture medium that allows growth of both types of microorganisms. For studies of *in vitro* antagonism on bacteria against phytopathogenic fungi is common to use the medium and Potato Dextrose Agar (PDA) (Lokesha and Benagi, 2007; Hu *et al*., 2008) that favors the growth of fungus. These types of tests allow the screening of potentially beneficial organisms to control plant pathogenic fungi. The selection of microorganisms with the inhibition of *Macrophomina* could be useful for evaluating the potential in greenhouse and/or open field production.

The aim of this study was to evaluate the antifungal activity of native strains of *Bacillus* sp. isolated from soil against *M. phaseolina* in dual bioassays using Nutritive Agar (NA), as this medium allowed growth of both types of microorganisms.

MATERIALS AND METHODS

Microorganisms: We used 13 native strains of *Bacillus* sp. presenting activity of chitinases (Rodas-Junco *et al*., 2009). These were isolated from different types of cultivated and uncultivated soils. The strains were maintained on NA at 4°C and were activated by seeding Petri dishes with nutrient agar and incubated 30°C for

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24-48 h. The strain of *M. phaseolina* was kindly donated by Dr. Mario Ramirez-Lepe from the Technological Institute of Veracruz (ITV, Mexico) and was maintained on PDA and then growth at 28°C, 72 h and stored at 4°C.

Dual test: A dual type bioassay was performed (Huang *et al*., 2005; Lokesha and Benagi, 2007) to determine the percentage of growth inhibition of *M. phaseolina* by strains of *Bacillus* sp. Seeding of the strains of *Bacillus* sp. and *M. phaseolina* was performed in a Petri plate with NA at 3 cm between them and the strains of *Bacillus* sp. and *M. phaseolina* in an individual as control, were incubated at 28°C for 6 days, the radial growth of microorganisms was followed and the inhibition percentage was calculated according to the formula reported by Lokesha and Benagi (2007).

Chitinases production: To obtain crude extract of chitinases, crushed shrimp shell was used as a source of chitin at 6% in distilled water, fermentation was performed at 28-30°C and 250 rpm for 120 h (Reyes-Ramirez *et al*., 2004), the crude extract was recovered by centrifugation at 1972×g, 5°C for 15 min. The enzyme activity was determined according to Monreal and Reese (1969) for the release of N-Acetyl Glucosamine (NAG) using colloidal chitin as substrate. One Unit of chitinases (U) was defined as the amount of enzyme that catalyzes the release of 1 mg of NAG/h at 50°C. The crude extract was sterilized with a 0.22 μm Millipore membrane (Millipore Corporation, Bedford, USA) to obtain a crude extract of free cells.

Effect of chitinase on *Macrophomina phaseolina*: The crude extract was adjusted to pH 6.0, to avoid growth inhibition by an alkaline pH. The crude extract was used to 10% on PDA, added to the medium before solidifying and dispensed into a petri plate was inoculated with a 5 mm disk of mycelium of *M. phaseolina* in the center and radial growth was recorded for 6 days. Phosphate buffer pH 6.0 was used as control. Five repetitions were performed. The results were analyzed by an Analysis of Variance (ANOVA) and Tukey's test ($p \leq 0.05$) using SPSS 15.0.1 for Windows (Lead Technologies, Inc. USA).

RESULTS AND DISCUSSION

The strains of *Bacillus* sp. used in this study were previously isolated from different soil types, cultivated and uncultivated in the district of Juquila, Oaxaca, Mexico. The selected strains produce chitinases and proteases (Rodas-Junco *et al*., 2009). Isolates of *Bacillus* sp. were made by Gram staining, catalase test and formation of endospores as confirmatory evidence of the genus *Bacillus* and the production of chitinases by the hydrolysis of halo formation in minimal medium with colloidal chitin according to Rodas-Junco *et al*. (2009). The dual test was conducted in petri plates with NA medium, the medium of choice is not the usual for the growth of fungi (PDA), however, showed a radial growth of *Macrophomina* of 54 mm to 120 h, similar to that achieved in the PDA at 72 h, despite a slower growth observed, was sufficient for both types of microorganisms and dual test (Fig. 1). The radial growth of microorganisms and the percentage of inhibition calculated in all the strains tested showed a decrease in growth of *M. phaseolina* and was observed a higher percentage of inhibition in the strains LUM B01 with a 80.8±6.5%; LUM B04 (75.9±3.8%); LUM B57 (68.5±1.5%); LUM B65 (66.3±1.5%) and LUM B19 (65.9±7.8%).

There was no significant difference ($p \leq 0.05$) between these strains (Fig. 2). All strains of the used *Bacillus* sp. showed a percentage inhibition >50% (range 54-80%), except for strain LUM B15 with a 31.1±1.5%. Values

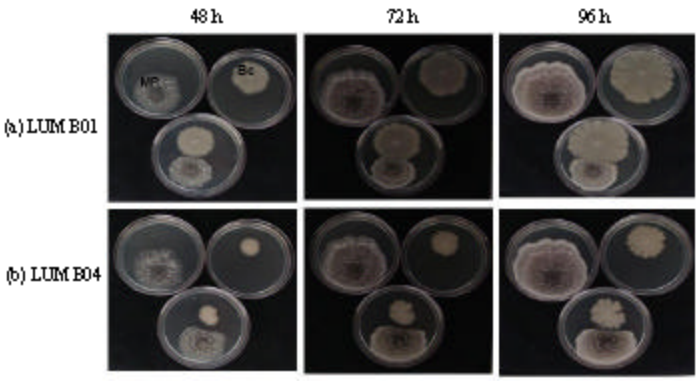

Fig. 1: Antifungal activity of native strains of *Bacillus* (a) LUM B01 and (b) LUM B04 on NA plates against *M. phaseolina*. MP: *M. pahaseolina*; Bc: *Bacillus* sp.

close to 75% inhibition of growth of *Macrophomina* by *Bacillus subtilis* were observed by Lokesha and Benagi (2007) using a PDA culture medium. These values are comparable to those observed in this research by strains LUM-B01 and B04. We observed a more rapid radial growth of the strain LUM B01, so in the dual test, reached the fungus at 72 h (Fig. 1), this could explain the higher percentage of inhibition of this strain due to competition between the two organisms for the available space in the petri plate. Once, they reached the contact with the fungus strain they followed the bacterial growth on the fungus inhibiting further growth of *Macrophomina*, possibly in a process of parasitism. This was observed in strains of *Bacillus* sp. that grew to reach the fungus. We observed the growth inhibition of *M. phaseolina* without contact with the bacterial strains (Fig. 1), this could be due to the production of hydrolytic enzymes or antibiotics extracellular compounds, which are disseminated through the media. In *B. subtilis* and *B. pumilus* have been identified antibiotics with antifungal activity (Leifert *et al*., 1995). To test the effect of hydrolytic enzymes on the growth of *Macrophomina*, we used a crude extract with activity of chitinases produced by fermentation using as a source of ground shrimp shell chitin. For the production of chitinases strains LUM B01 and B04 were used, for presenting the highest percentage of *in vitro* inhibition of *M. phaseolina*. The fermentation was conducted at 120 h as it is the maximum time reported for production of chitinases of *Bacillus* strains using shrimp wastes (Reyes-Ramirez *et al*., 2004), both strains were producing chitinases (Fig. 3). The production of chitinases was significantly higher in the strain LUM B04 at 96 h, but at 120 h of fermentation there is no significant difference being 0.435±0.035 U mL^{-1} for LUM B01 and 0575±0.007 U mL^{-1} for LUM B04. Possibly this is due to a decrease in the production of chitinases around 102 h due to the presence of proteases in the medium (Reyes-Ramirez *et al*., 2004). To determine the effect of chitinases on the growth of *M. phaseolina*, crude extract of the strain LUM B04 of 120 h of fermentation was used, because it presented the highest production of chitinases. There was a significant decrease of radial growth from the 48 h, the crude extract showed a decrease of nearly 30% compared with the control (Fig. 4). A nearly 100% decrease reported in *Sclerotium rolfsii* using a crude extract of chitinases concentrated by ultrafiltration, the difference in the rate of growth varies according to different types of species are reported from 18-90% and the concentration of activity enzyme used (Reyes-Ramirez *et al*., 2004). The slowdown in the growth

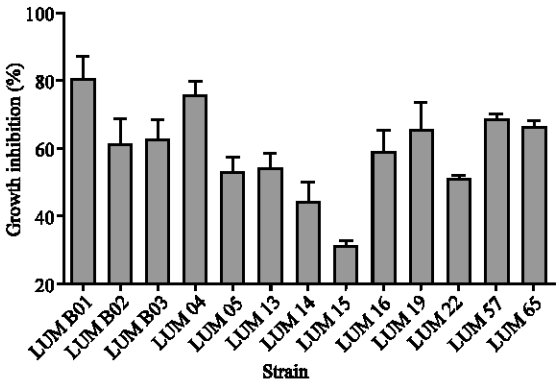

Fig. 2: *In vitro* inhibition of growth of *M. phaseolina* by *Bacillus* sp.

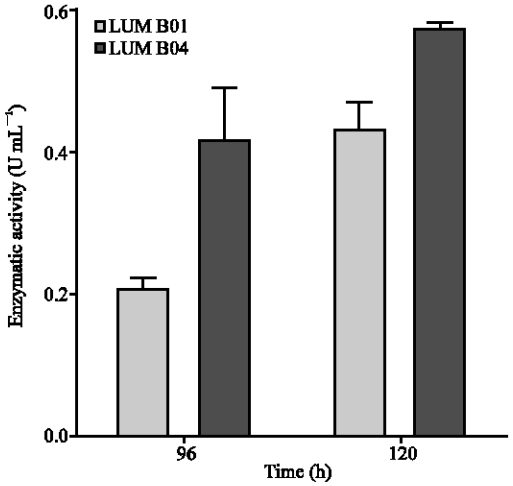

Fig. 3: Chitinolytic activity of LUM B01 and LUM B04 growing on shrimp wastes

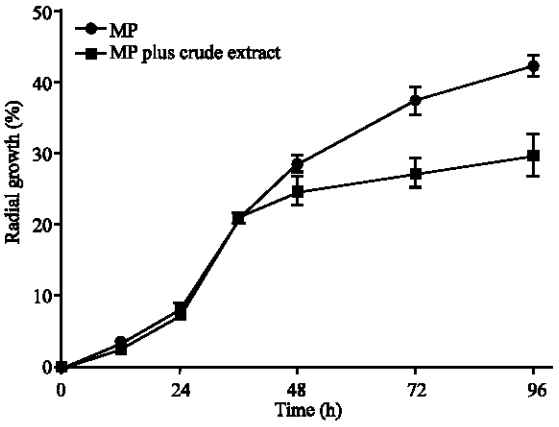

Fig. 4: Effect of crude chitinases extract from *Bacillus* sp. LUMB 04 on the growth of *M. phaseolina* on PDA plates

of *M. phaseolina* is due to the presence of chitinases in the environment, however there may be involvement of other enzymes such as β-1-3 glucanase, as well as other molecules such as antibiotics that may involved in the growth inhibition of phytopathogenic fungi (Leifert *et al*., 1995). Chitinolytic microorganisms are considered as having the potential for biocontrol of phytopathogenic soil for their antagonistic ability.

CONCLUSION

Dual assay method allows determining antifungal activity of the native strains of *Bacillus* sp., showing higher activity of strains LUM B01 and B04. Hydrolytic enzymes affect the growth of *M. phaseolina*. The native strains of *Bacillus* sp. LUM B01 and B04 are chitinolytic microorganisms with antifungal activity, could be evaluated *in vivo* to test the potential to be used in biological control of plant pathogenic fungi.

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