ANTIFUNGAL ACTIVITY OF *Bacillus* sp. E164 AGAINST *Bipolaris sorokiniana*

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ABSTRACT

Spot blotch caused by *Bipolaris sorokiniana* is an important disease of wheat in warmer and humid weather conditions, whose control relies mainly on chemical antifungal agents. The purpose of this work were to isolate bacteria strains with antifungal activity against *B. sorokiniana*; to select the best inhibitor and to evaluate the best growth conditions for the antifungal production; and to test it´s action *in vivo*. The bacterial strains were pre-screened against four *B. sorokinian*a isolates on plates containing Sabouraud maltose agar. The isolate that showed the best result was grown on different culture media, cells were filtrated and the filtrates were tested against *B.sorokiniana* on plates with PDA medium. The *in vivo* test was done on wheat seeds, infected with *B. sorokiniana* isolate on a chamber with controlled temperature. *Bacillus* E164 was chosen among the 86 bacterial isolates tested against the phytopathogen. The filtrate from *Bacillus* E164 grown on tryptic casein soy broth (TSB) and straw culture media showed a similar degree of inhibition against the phytopathogen, the same result was not observed with malt extract broth media. The filtrate culture showed activity similar to control being submitted to temperatures from 50- 90ºC. The *in vivo* test with isolate E164 caused morphological effects on wheat plants with a significant root length reduction. Control exerted by *Bacillus* E164 over *B. sorokiniana* isolates was relevant *in vitro*, nevertheless the influence and importance of the metabolites produced must be elucidated for the application *in vivo.*

Key-words: *Bipolaris sorokiniana*, *Bacillus* sp., biological control, antifungal activity.

ATIVIDADE ANTIFUNGICA DE *Bacillus* E 164 CONTRA *Bipolaris sorokiniana*

RESUMO

Mancha marrom causada por *Bipolaris sorokiniana* é uma importante moléstia do trigo em países onde as condições climáticas são de alta umidade e calor. O controle da moléstia baseia-se principalmente em fungicidas. O presente estudo teve como objetivos: isolar cepas bacterianas com atividade antifúngica contra *B. sorokiniana;* selecionar o melhor o melhor isolado com capacidade de inibição e avaliar as melhores condições de crescimento para a produção do antifúngico; e testar a sua ação *in vivo*. As cepas bacterianas foram pré-selecionadas contra quatro isolados de *B. sorokiniana* em placas de Petri contendo agar Sabouraud maltose. A bactéria com a melhor ação antagonista foi crescida em diferentes meios de cultura, as células foram filtradas e os filtrados foram testados contra *B. sorokiniana* em placas contendo meio agar batata dextrose (BDA). Também foi avaliada a estabilidade do(s) metabólito(s) em diferentes condições de temperatura e pH. O teste *in vivo* do antagonista foi realizado contra um isolado do fitopatógeno em câmara com temperatura controlada. O isolado de *Bacillus* E164 foi escolhido entre 86 isolados bacterianos testados. O filtrado de *Bacillus* E164 crescido em caldo tripticaseína de soja e em caldo de palha de milho mostrou resultados similares de inibição O mesmo resultado não foi observado quando a bactéria foi crescida em caldo de extrato de malte. O filtrado mostrou atividade similar ao controle após ser submetido às temperaturas de 50 a 90 ºC. O teste *in vivo* com o isolado E164 causou efeitos morfológicos nas plantas de trigo causando uma reduzida significativa no comprimento das raízes. O controle que o *Bacillus* E164 exerceu contra *B. sorokiniana* foi relevante apenas *in vitro*, no entanto, a importância dos metabólitos produzidos precisa ser elucidada para a sua aplicação de forma mais eficiente *in vivo.*

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INTRODUCTION

Bipolaris sorokiniana (Sacc. in Sorok.) Shoemaker. telomorph: *Cochliobolus sativus* (Ito & Kuribayashi) Dredchs. ex Dastur, is a worldwide pathogen of wheat and other cereal grasses which can infect seeds, leaves and roots. The diseases caused by this phytopatogen are common root rot, spot blotch, seedling blight and black point of the grain. The main source of inoculums is considered the soil-borne conidia, which under favorable conditions, may result in severe common root rot disease (DUCZEK et al., 1996).

In Brazil, fungal diseases specially affect wheat, a main constraint to its cultivation (METHA, 1978). Brazilian wheat production is insufficient to supply internal demand CONAB (2005), with losses reaching 80% (REIS et al., 1995). There are no efficient fungicides to control the disease and there is a lack in cultivar resistance (METHA, 1978). By combining fungicide treatment and crop rotation, this control is partly achieved accordingly Reunião (2004). However, the need to minimize the release of pesticides to the environment in a sustainable agriculture context require a better understanding of gentotype *versus* environment interactions of this disease and so a management of adequate strategies will be possible.

The use of antagonist bacteria or preparations, made therefore, is an efficient means for biological suppression of soil phytopathogens that cause root rot (SHIROKOV et al., 2002). Researches *in vitro* using antagonist microorganisms applied on seed and foliar surfaces have shown very good inhibition results (KOMMEDAHL AND MEW, 1975; LEIFERT et al., 1995; ZANHG AND YUEN, 2000a, 2000b].

Secondary metabolites produced by some *Bacillus* strains have shown antibacterial and antifungal activity against phytopathogens and food spoilage microorganisms (BERNAL et al., 2002; CZACZYK et al., 2000; FÖLDES et al., 2000; MCKEEN et al., 1986; MILNER et al., 1996; PICHARD et al., 1995; SHIROKOV et al., 2002; TOURÉ et al., 2004). The sporulating Gram-positive bacteria offer biological solutions to the formulation problems, with heat and dissection resistant spores been prepared readily into stable products (Emmert and Handelsman, 1999). Therefore the objectives of this study were: 1) to select and evaluate the best bacterial isolate with antifungal activity against *B. sorokiniana*; 2) to optimize culture conditions for production of the metabolite(s); 3) to improve knowledge of physical-chemical properties of the metabolite(s); and 4) to test the effects of the antagonist isolate against one *B. sorokiniana* strain *in vivo*.

MATERIALS AND METHODS

***B. sorokiniana* isolates:** *B. sorokiniana* strains 98003, 98004, 98006, 98007, 98010, 98011, 98012, 98013, 98014, 98017, 98018, 98019, 98021, 98022, 98023, 98025, 98026, 98028, 98029, 98030, 98031, 98032, 98033, 98034, 98041, 98042, 98043, 1992/19, CEV13, CEV48, CEV53, M05, A20 and NRRL were purchased from the collection of our laboratory and CYMMIT (México). Strains were maintained on potato dextrose agar (PDA) at 4°C.

Isolation of bacterial isolates: Seeds from wheat cultivars CEP24; CEP30; BR26; OR1; BR35; E119; Rubi; BRS49; BR18; BRS177; E16; BR23; CEP31; BRS120; E40 and RS15 were supplied by Centro Nacional de Pesquisa de Trigo (EMBRAPA/CNTP), Passo Fundo, RS, and Fundação Estadual de Pesquisas Agronômicas (FEPAGRO), Londrina, PR. Seeds from all cultivars were used for the isolation of bacteria isolates.

Screening of bacteria isolates with activity against *B. sorokiniana*: Twenty seeds of each cultivar were macerated, mixed with 10mL of sterile distilled water. The suspension was agitated, twice, for 2-min in a vortex with a rest period of 5-min between the agitations. Afterwards it was serially diluted and from each dilution, 1mL was seeded onto plates together with 15 mL of tryptic soy agar medium (TSA) using the pour plate method. Incubation took place at 24°C for 24-48h. After incubation, colonies were purified and cultures maintained in TSA slants tubes at 4ºC.

***In vitro* antibiosis assay:** Eighty-six bacterial isolates were used in the antibiosis assay against four *B. sorokiniana* (89017, 98031, 98042, 1992/19) isolates. Bacteria isolates were inoculated on 2mL of tryptic soy broth medium (TSB), incubated at 24°C for 24h. The cultures were used to inoculate two plates each, containing Sabouraud-maltose, and incubated as before. After growth, 5mL of sterile distilled water was poured onto plates mixed with the cells, and 2mL of each suspension transferred onto plates with Sabouraud-maltose and incubated as before. Subsequently, each isolate was transferred to a new plates with the same medium, by gently patting the rim of a 5cm sterilized glass funnel onto the culture surface, and than onto the plates. After 24h of incubation, a 0,7cm disc containing pathogen mycelium was placed on the center of the blot formed by the bacterium culture. Each dual culture, pathogen-antagonist, had five repetitions. A control plate with Sabouraud maltose containing only the disk with the pathogen mycelium was prepared for each set of five plates. Plates were incubated at

24°C with a 12h photoperiod for five days. During incubation, measures of fungal growth were taken, and the proportion of fungal mycelium growth, under bacterial control, was marked down against fungal growth on control plate (LUZ, 1990).

Establishment of growth intervals: Each bacterial isolate was labeled as efficient (E), moderately efficient (ME) and inefficient (I), against each *B. sorokiniana* isolate, according to the following growth percentage criteria of the phytopathogen: from zero to 20%; > 20 to ≤ 50%; and > 50 to ≤ 100%, respectively.

***In vitro* inhibition assay of three bacteria isolates against *B. sorokiniana* strains:** Fungal isolates grown on plates with PDA medium for 7 days were used to prepare conidial suspension. Colonies mixed with sterile saline solution were transfer into sterile glass tube and conidia final concentration adjusted to $5x10^5$ conidia/mL. 1mL of the conidia suspension, mixed with 9mL of melted PDA medium were homogenized and poured onto plates. After solidification of the medium bacterial strains, with 24h of growth, were inoculated with a needle on three equidistant points onto the plates. Incubation took place at 25 ºC for 5 days. After incubation, plates were evaluated for antagonistic activity, considering the ability of the microorganism to reduce pathogen expansion, haloes were measured.

Conidial germination assay: *B. sorokiniana* isolates used for this assay were 98017, 98031, 98032, and 1992/19. The assay followed the method described by Lorito et al.(1993) with modifications. Bacterial strains were cultivated under agitation on TSB medium at 37°C for 24h, and after incubation, centrifugation took place at 10,000rpm for 8min and the supernatant collected. In a microcentrifuge tube, a mixture was prepared with the same volume of conidial suspension, potato dextrose broth (concentrated 3 times) and bacterial supernatant. In the negative controls, the same volume of saline 0.85% substituted the bacterial supernatant. Incubation of the mixture took place at 25 º C, and after 22 and 28 hours conidia, samples were stained with lactophenol blue, visualized under light microscope.

Bacteria identification: The bacteria tested and established as efficient antagonist against *B. sorokiniana* underwent standard microbiological identification routines. The examination started with Gram staining, analysis of cell shape and motility under microscopy. Biochemical tests were used according to the traditional methods: as oxidation/fermentation reaction, presence of catalase, and oxidase enzymes, growth on nutrient broth at different temperatures, assimilation of different carbon and nitrogen sources, hydrolysis of gelatine, starch, casein and others (SNEATH et al., 1986; MACFADDIN, (2000).

Assay of bacterial growth in different media: Bacteria strain was grown on TSB, malt extract – ME (malt extract 30g/L; soy peptone 3g/L) and corn straw based media –CS (soy peptone 15g/L, sodium cloride1g/L, dextrose 2g/L, yeast extract 5g/L, corn straw 5g/L) at 37ºC with agitation for 48 hours. The cultures were filtrated with a 0.22µm filter and diluted 5, 25 and 50 times with distilled water. From each dilution, one aliquot of 50µL was poured into 5mm diameter wells, previously prepared on plates with PDA medium. Incubation took place first under refrigeration for 16h for better diffusion of the filtrate, and afterwards at 25ºC for 5 days when the inhibition haloes was measured. Tests were done in duplicate and repeated three times.

***In vivo* root control: C**ulture from the bacterium isolate grown in TSB for 48h was centrifuged, cells collected, and the concentration standardized to 10^8 cells/mL, using Mac Farllan scale, and cells used for soil inoculation. *B. sorokiniana* isolate 98031 was grown on PDA medium for 6 days, and a suspension of 10^6 conidia/mL was prepared and used for seed contamination. Healthy seeds, from wheat cultivar 209 EMBRAPA-TRIGO, Passo Fundo, Brazil were contaminated with the conidia suspension (10µL/seed) incubated in a moisture chamber at 25 º C for 5 days. Twelve pots containing autoclaved sand, vermiculite (1:1) and NPK solution (30:10:10) were sown with four contaminated seeds each. Six pots only with the contaminated seeds were positive control; in the other six pots 20mL of bacterial suspension was added. Seeds, without *B. sorokiniana* contamination, were sown on six pots and used as negative control. Incubation took place in a chamber at 20ºC with 14h photoperiod and periodical irrigation with distilled water. After 21 days, plants were collected and rhizosphere soil washed out with sterile distilled water. Length of the second leaf and roots measured and the presence of lesions or disease symptoms was evaluated.

Root analysis: Ten roots from the negative and positive control and six roots from the pathogen x antagonism assay were cut in two parts. One part placed on plates with PDA medium to confirm the *B. sorokiniana* infection and a second part clarified and stained using the Phillips & Hayman (1990) method with modifications. The roots were treated with a 10% KOH solution at 100ºC for 15 min, washed twice with distilled water and socked in FAA solution (chloroform 10%, acetic acid and ethanol 50%, 1:1:18). Staining was done using

acetic acid 1M, glycerol and water (1:1:1) with toluidin blue 0.05%. The presence of conidia or other structures were evaluated under the microscope.

Thermal stability of bioactive metabolite: Different temperatures were used in this assay to evaluate the thermal stability of the metabolite(s), produced by the bacteria. Samples of the filtrated bacterial culture were incubated in a water-bath with temperatures varying from 50ºC to 100ºC for 30, 60 and 90 minutes. After incubation 50µL of each sample were poured into wells, previously prepared in plates with PDA medium containing fungal spore suspension of *B. sorokiniana* isolates used in previous assays. Filtrate without heat treatment used as control. Plates were left overnight under refrigeration, for a better diffusion of the filtrate, and than incubated at 24ºC for 5 days. Inhibition haloes measured after incubation. The same procedure was done using lower temperature, where samples were kept under refrigeration 4 ºC and -20ºC for 1, 5, 7, 14 and 21 days. Incubation conditions were as described before. Experiments were repeated twice.

Filtrate stability under pH variations: Buffer solutions were prepared on PD broth (potato dextrose broth). Disodium phosphate buffered with citric acid was used for pH 4.0 to 8.0, and the same salt was buffered with sodium hydroxide for pH 9.0 to 10.0. Conidia, from isolate 98031, grown on PD medium were used in this assay. In glass tubes, a mixture with 4mL of each buffer 0.9mL of the filtrate bacteria culture and 0.1mL of conidial suspension (10^6conidia/mL) was prepared and incubated at 25ºC for 10 days. The filtrated culture was replaced by sterile TSB in the control tubes. Samples were filtrated and dried biomass obtained by placing the filters at 100^0C upon constant weight.

Statistical analysis: Statistical analysis of the data was performed using the Analyse-it + General Program for Microsoft Ecxel, release 1.73. Analysis of variance was used to analyze the effects of inhibition tests *in vitro* and activity for the bacterial filtrated culture after all treatments. Scheffe's Test used to investigate the significance of cross comparisons.

RESULTS

The results obtained with the screening 150 bacteria were isolated from the wheat seeds. The *in vitro* antibiosis assays of bacterial isolates against *B. sorokiniana* strains showed that 43 (49. 4%) of the isolates were inefficient (I) as antagonists against the phytopathogen, 39 (44.8%) were moderately efficient (ME), and 5 (5.74%) isolates were efficient (E) producing metabolites which inhibited *B. sorokiniana* growth.

Isolates from cultivars OR1, BR35 and BRS177, were most potentially antagonistic against *B. sorokiniana* isolates. The average of growth data of each phytopathogen underwent the variance analysis (ANOVA) with $p \leq 0.05$. A matrix was, thereby generated, comparing *B. sorokiniana* growth within each wheat cultivar. The results showed no statistically significant difference for the average growth between fungal isolates 98031, 98032, 98017 and 019/92 antagonized by bacterial isolates from cultivar OR1, BR35 and BRS177.

The strains that showed efficient results of inhibition against *B. sorokiniana* strains were submitted to biochemical tests. All samples were Gram-positive rods with endospores. The biochemical tests done with the isolates confirmed the genus *Bacillus*.

The three *Bacillus* sp. strains E164 (BR35), 3OR1 (OR1) and 2BRS (BRS177) were used in the *in vitro* assay against the 34 *B. sorokinina* isolates. All of them had shown inhibitory activity against the phytopathogen isolates. Significant differences, among inhibition haloes, were observed for *Bacillus* E164 and *Bacillus* 3OR1 when compared to *Bacillus* 2BRS (Table 1).

When the inhibition of each *B. sorokiniana* isolate was analyzed individually (Scheffe's $p<0.0001$), *Bacillus* sp. 3OR1 presented better activity against isolates 98031, CEV53 and 1992/19. *Bacillus* sp. 2BRS showed better results over 98013 and 98043, whereas *Bacillus* sp. E164 inhibition was better against 98007, 98010, 98012, 98013, 98014, 98030, CEV48 and CEV13. Significant differences were observed among fungal isolates inhibited by *Bacillus* 2BRS while a more homogenous inhibition was obtained with *Bacillus* E164 and 3OR1. Therefore, the *Bacillus* sp. E164 was the isolate chosen for further studies in this work.

Different culture media (TSB, ME, CS) were used to grow *Bacillus* sp. E164. After growth, the cultures were filtrated and tested against five isolates of *B. sorokiniana* (98017, 98022, 98025, 98042 and 1992/19). The antifungal production was lower on ME medium when compared with the production on TSB, or CS media. A significant difference was observed among the inhibition haloes produced with the filtrated cultures, against the five phytopatogens, from filtrate cultures on TSB/CS and ME (Table 2).

The minimal inhibitory concentration (MIC) was determined with filtrate culture of the *Bacillus* E164 grown on TSB medium. The results obtained with different concentrations of the filtrate, tested on plates against the *B. sorokiniana* strains, zones of inhibition growth of the phytopathogen

were observed at 10 times dilution of the crude extract.

Cultures of bacterial *Bacillus* E164, 3OR1 and 2BRS were centrifuged and the supernatant mixed in separate tubes with conidia from each of the five isolates of *B. sorokiniana*. The effects of supernatant over conidial germination and hyphae developments were similar for all the strains of the phytopatogen. Germ-tubes of conidia showed morphological alterations: they were partially lysated, the hyphae had granular and vacuolated cytoplasm and abnormal mycelia development (Figure 1).

Filtrate was submitted to a thermal treatment in order to evaluate the stability of the antifungal under different temperatures. The antifungal activity kept constant when the filtrate was submitted to temperatures from 50-90ºC, showing an inhibition activity similar to the control (Figure 2). There was a significant loss of activity at temperature of 100ºC ($F = 11.7$; $p < 0.0001$; $n = 42$) when compared to the control and other treatments. Even though, at this temperature, 30% of activity was observed.

The stability of the filtrate was tested on different pH treatments varying from pH 4.0 to 10.0. Analyzing the mycelium dry weight it was possible to observe a decrease in mycelia growth as pH increased (Figure 3). The statistical analysis of control mycelium dry weight, and mycelia dry weight from pH treatments, a significant difference was observed from control and pH 5.0, 6.0, 8.0 and 10.0 ($F = 4118,3$; $p < 0,0001$; $n = 28$).

The *in vivo* experiment showed that when the soil was inoculated with the filtrate of antagonistic bacteria, the wheat cultures presented more leaves, ticker and shorter roots compared to those of the control (Figure 4). From the 44 leaves analyzed, only three of them showed lesions typical from *B.sorokiniana.* Two of these leaves were from the wheat plants grown on substrate without the bacteria and one was from a sample where the antagonist has been tested. Leaf lengths were similar among treatments. All the roots presented filamentous fungal contamination. However, these contaminations were restricted to the epidermal surface, as inner tissues were not reached. Roots derived from seeds infected by *B. sorokiniana* presented conidia on epidermal surface.

Distinct fungal populations could be observed after the roots were placed on agar plates and incubated at 25°C. Roots obtained from seed not contaminated (negative control) the presence of *Alternaria* sp., *Curvularia* sp., *Fusarium* sp., *Penicillium* sp., *Epicoccum* sp. or *Aspergillus* sp was detected. On the other hand, those roots from contaminated seeds only the presence of *B. sorokiniana* was observed.

DISCUSSION

Biological control using microorganisms to suppress plant diseases offers a powerful and environmentally friendly alternative to the use of synthetic pesticides. No durable resistance to the diseases caused by *B. sorokiniana* currently exists. The control relies on an integrated combination of good soil nutrition as part of crop management approach (SHARMA et al., 2006; REUNIÃO, 2004), fungicides (CONNER & KUZYK, 1988; REUNIÃO, 2004).

Even though efficient results were obtained in the *in vitro* inhibition of fungal growth, this method cannot be the only one to be used when choosing the antagonistic bacterial agent (WELLER et al., 1985). Experimental results of studies performed in greenhouses point to the fact that biologic control agents that show *in vitro* antibiosis activity do not necessarily behave in such way when *in vivo* assays are performed (PERONDI et al., 1995; MÓNACO et al., 2004; PERELLÓ et al., 2003). Combinations of isolates may also bear an advantage over the use of isolates alone by wielding therefore a synergistic action on the phytopathogens.

The antagonistic phenomenon between microorganisms occurs naturally in soil, around the root surface (rhizosphere), stem and leaves, and is easily observed in pathogens cultures, when contamination happens in laboratory (ODIGIE & IKOTUM, 1982). Probably, fungistats or fungicides able to spread throughout a given medium must be involved in this antagonistic action.

The advantages of crop treatments with fungicides are reliable, simple handling, and of low costs. Notwithstanding, under long periods of low soil temperatures and high humidity, microorganisms may offer results against fungi, which outdo those of fungicides. This behavior is probably due to the growth of those microorganisms within the seeds that, when the plant shoots forth, eventually reach the root surface, protecting the tissues. Therefore, research is still needed: 1) on the effects of temperature and humidity of soil; 2) the effects on length of time that seeds are held in stock after treatment; 3) in the use of several microorganisms sequentially or as a mixture; 4) toxicity for animals; 5) and adverse effects on the environment to fully establish possible antagonists. The use of microorganisms to protect seeds should be commercially feasible as soon as such research develops more integrally. Some studies have already shown the viability of biological control against root

diseases in crops by protecting seeds with the help of antagonistic microorganisms (KOMMEDAHL & MEW, 1975).

For many years, the genus *Bacillus* has been studied due to its capacity to produce many metabolites, which can be used in biological control or antibiotics production. Formulations of *Bacillus subtillis*, already commercialized, have been used with a good efficacy of protection against *Fusarium* and *Rizoctonia*, in which they also stimulate the growth of the plants (EMMERT & HANDELSMAN, 1999).

The inhibitory potential and antibiotic production of *Bacillus subtillis* was investigated *in vitro* against phytopathogen fungus and bacteria (FÖLDES et al., 2000). Touré et al. (2004) attributed to lipopeptides produced by one isolate of *Bacillus subtillis*, the antagonistic action over mycelia growth, which they obtained over many phytopatogenic fungi from soil, as *Fusarium, Pythium ultimum, Rizoctonia solani* and *Rhizopus*.

According to Handelsman & Stabb (1996) it is common for bacteria, which are investigated for the use as biological control, to produce more than one antibiotic. Therefore, each strain may have distinct activity over the structures of the fungi.

Considering the differential, on inhibitory efficiency, among the bacteria isolates in this work, the choice for *Bacillus* E164 was completely random since it had similar results with the isolate 3OR1. The results obtained, using the filtrate from *Bacillus* E164 culture against all *B. sorokiniana* strains was compatible with results observed by Leifert et al. (1995), where the authors got inhibition of *Botrytis cinera* using only the fermented broth from *B. subtillis* cultivation. Similar works obtained good results in antagonism between *Bacillus* strains and fungal pathogens, including *B. sorokiniana* (BERNAL et al., 2002; PERELLÓ et al., 2002; SHIROKOV et al., 2002; SMITH et al., 1999).

The result obtained with different carbon sources was not expected. Because, the filtrate obtained from the cultivation, using maize straw and malt extract medium, showed a lower inhibition than with TSB, and these substrates are already part of the natural biota of the microorganisms. It was expected that with substrates, with more natural composition, the metabolite(s) production would be better than with TSB. Many authors (MILNER et al., 1996; CZACZYK *et al.*, 2000; NILSEN et al., 1999) obtained a much better result with more natural carbon sources.

The metabolite(s) produced by *Bacillus* E164 showed a very good stability at high temperatures. This result differs from the ones obtained by Shirokov et al. (2002) were 60% of the activity of the filtrate was lost when it was submitted to 70 ºC. Many microorganisms have an intrinsic capacity to produce secondary metabolites *in vitro* (excluding siderophores and enzymes), and there is evidence of their relevant action *in situ* (RAAIJMAKERS et al. 2002, TOURÉ et al. 2004).

The differences in susceptibility, observed for each fungal isolate, in relation to the antagonist action of *Bacillus* E164 may be related to variations in the production of inhibitory metabolites by the bacterial strains. Resistance and resilience mechanisms may be involved too, as each *B. sorokiniana* strain presents different behavior, when antagonized by the same bacterial isolate. The effects of filtrates over conidia germination of *B. sorokiniana* strains suggest a deficient formation of cell wall with a vacuolization of hyphae and the mycelium with a torulose aspect, similar result obtained by Perelló et al. (2003) and Mónaco et al. (2004) studying the effects of *Trichoderma* against *Pyrenophora tritici-repentis* and *Bipolaris sorokiniana* respectively. This results obtained with the filtrate needs more investigations because many enzymes act over cell wall in different stages of its formation. Essentially, the action of antibiotics has been associated to inhibition of spores germination or germ-tube elongation, and the efficacy of biological control *in vivo* depends on the inhibition level of the pathogen at earlier phases of host infection (ZHANG & YUEN, 1999).

In vivo experiments showed no significant control of the disease and other workers using suppressor's bacteria (PERONDI et al. 1995, KOMMEDAHL & MEW 1975) observed similar results. The lack of disease reduction may be attributed to the low levels of disease seen even on positive controls (RYAN & KINKEL, 1997). On the other hand, an increase in bacterial population permitted a better plant development and this event can be related to production of phythormone-like molecules by bacterial.

Antagonistic action of *Bacillus* strains against *B. sorokiniana* have been observed by many workers (PERELLÓ et al., 2002, SHIROKOV et al., 2002, CZACZYK et al., 2000) however, none were done *in vivo*.

Experiments utilizing *Bacillus* sp. as antagonists are inconclusive with respect to the efficacy of this microorganism on field conditions, as shown by the low commercial progress of antagonists derived products (RAAIJMAKERS et al., 2002; SPADARO & GULINO, 2005). There is an agreement that endospore-forming bacteria are advantageous because of formulation facilities (EMMERT &
HANDELSMAN, 1999; SPADARO & GULINO, 2005) and resistance to the soil conditions.

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Table 01: Analysis of variance of inhibition haloes from in vitro antagonism among bacterial strains E164, 3OR1 and 2BRS against 34 *B. sorokiniana* isolates[1]

Source of variation	SSq	DF	MSq	F	P
Bacterial isolates (BI)	25,32	2	12,66	23,58	<0,0001
Fungal isolates (FI)	158,54	33	5,87	1,94	<0,0001
BI x FI	71,89	66	1,33	3,63	<0,0001
Haloes	164,32	372	0,54		
Total	348,18	407			

[1] SSq = sum of squares; DF = degrees of freedom; MSq = mean of squares (n = 408).

Table 02: Analysis of variance of inhibition haloes from *in vitro* assays among filtrated culture from *Bacillus* E164 grown on tryptic soy broth, malt extract and corn straw based media over five isolates of *B. sorokiniana*[1]

Source of variation	SSq	DF	MSq	F	P
Among media	1,66	2	0,83	10,29	0,0002
Among haloes	4,04	50	0,081		
Total	5,07	52			

[1] n_{total} = 53; tryptic soy broth n = 23; malt extract n = 13; corn straw based media n = 17. SSq = sum of squares; DF = degrees of freedom; MSq = mean of squares.

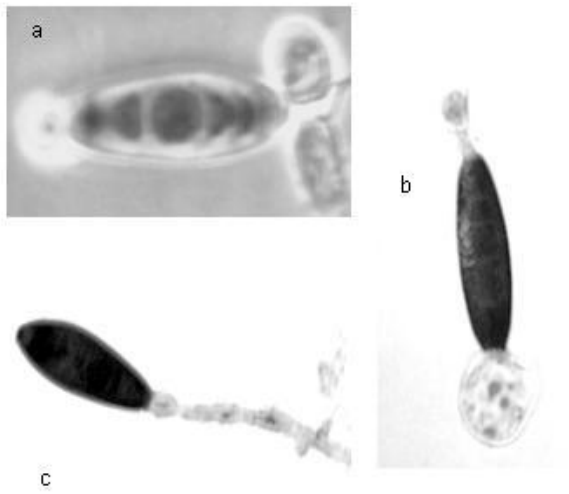

Figure 1: Conidia of *B. sorokiniana*, after 28 h incubation with the filtrated from *Bacillus* E164 grown in TSB medium- (left) isolate 98017 without supernatant from *Bacillus* E164; (right) 98017 with supernatant from *Bacillus* E164.

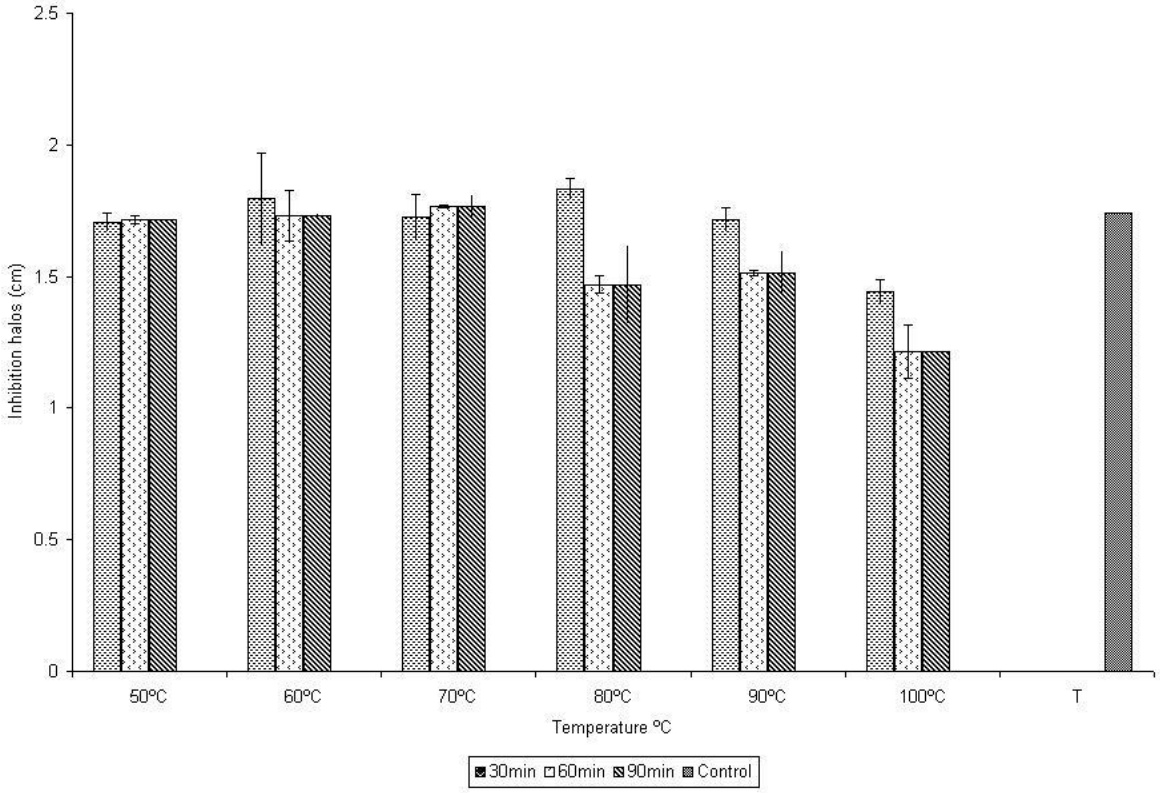

Figure 2: Activity of antifungal filtrated of *Bacillus* E164, after thermal treatments, against *B. sorokiniana* 98031. Time of treatments was 30, 60 and 90 minutes in different temperatures. T = control, environmental temperature (25°C).

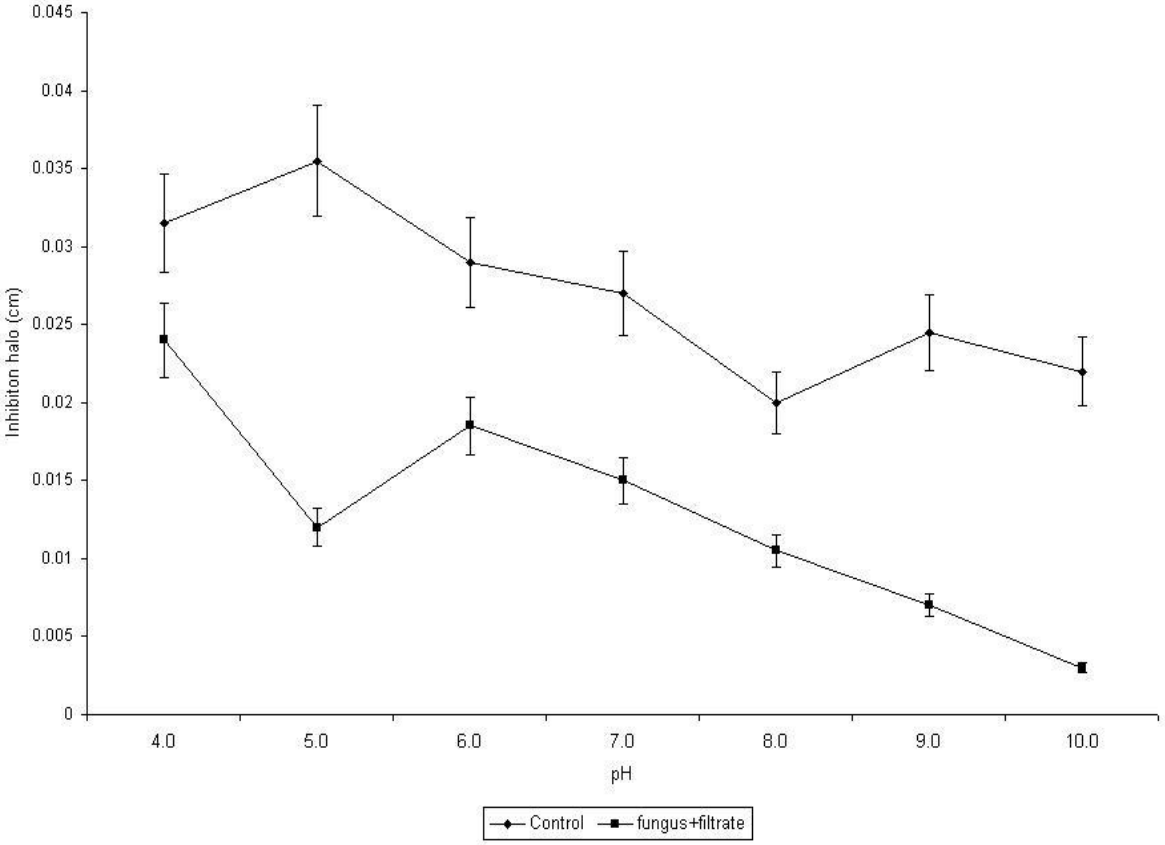

Figure 3: Stability of the filtrate from *Bacillus* E164 after treatment different pH. Dry mycelia mass obtained from *B. sorokiniana* 98031 grown in acid and alkaline potato dextrose broth with (∗) and without (□) filtrated culture from *Bacillus* E164

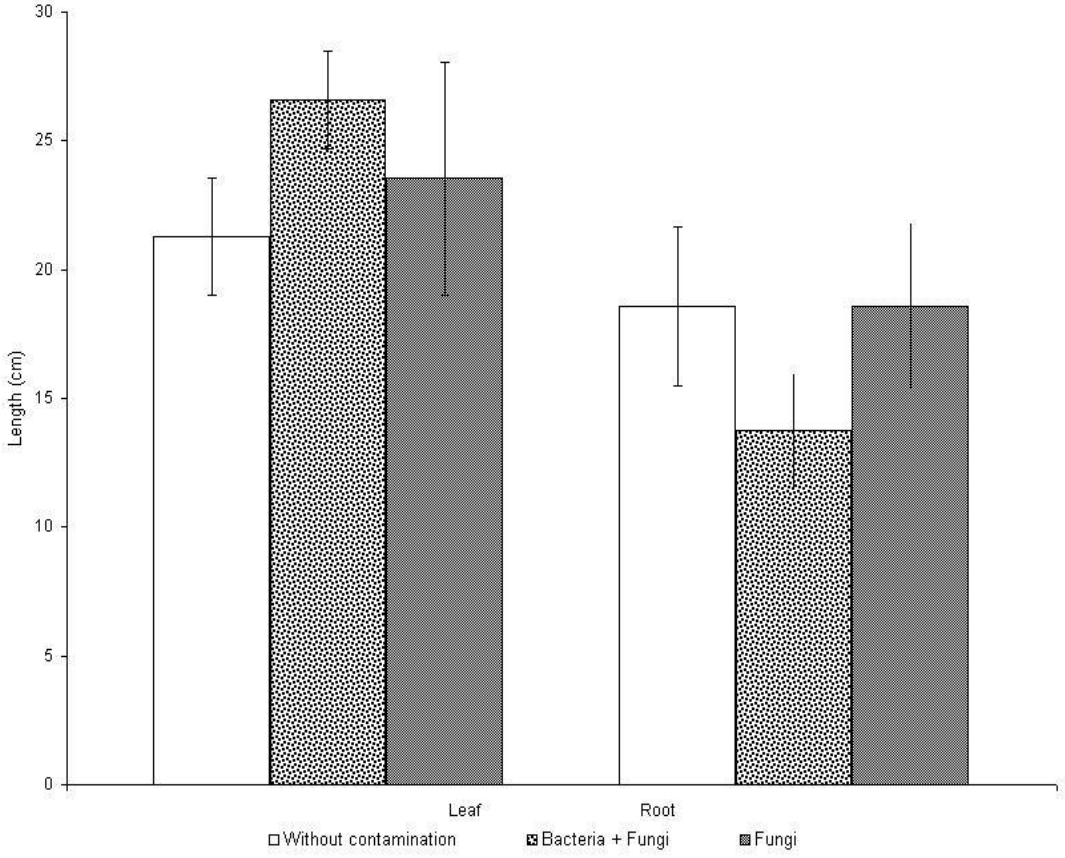

Figure 4: *In vivo* assay. Leaf and root length of wheat plants infected by *B. sorokiniana* treated or not with the antagonist *Bacillus* E164 after 21 days of growth in germinator chamber.